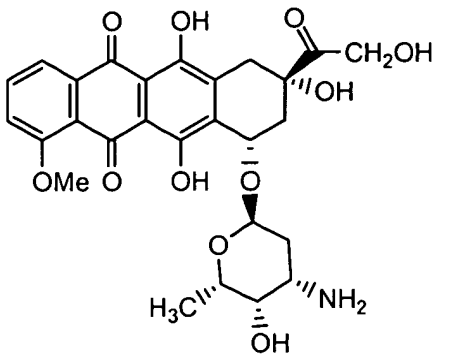
Remarks

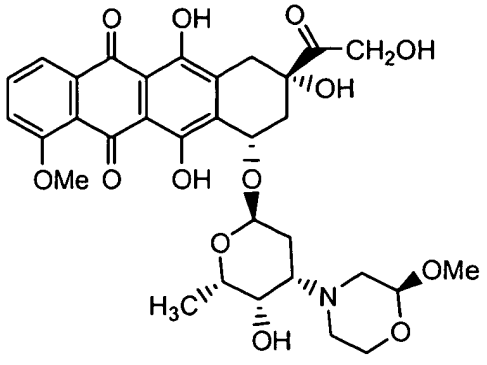
Claims 13-14, 18-19 and 20-31 remain rejected under 35 U.S.C. § 103 as being unpatentable over Kuhl et al. in combination with Miura et al and Gorbunova. Specifically, Miura teaches the treatment of liver tumors or hepatocellular carcinomas via hepatic artery administration of epirubicin, mitomycin C and lipiodol to decrease tumor volume or cause remission.[1] Kuhl teaches that MMDX is a doxorubicin analog that has the same tumor specificity as doxorubicin in CEM and K562 cells, and also is activated in the liver to a metabolite whose potency is 10 times greater. Gorbunova discloses that the use of intrahepatic areterial infusion chemotherapy allows for higher concentrations of an antitumor agent in the organ affected by the tumor. The Examiner contends that a person skilled in the art "would have been motivated to use MMDX in hepatic artery administration since the prior art recognizes that hepatic artery administration of doxorubicin is beneficial for decreasing tumor volume within hepatic carcinomas and reducing systemic exposure via direct administration to the organ; moreover, one of skill in the art would have a reasonable expectation of success in the use of a more potent analog of doxorubin, MMDX, in the same method of treatment." Applicants respectfully disagree with the obviousness rejection for the following reasons.

First, while MMDX is labeled as an "analog" of doxorubicin, the two are distinct chemical entities. Specifically, doxorubicin has a terminal amino group at the 3' position of the sugar moiety, while MMDX has a 3-methoxymorpholino group at the 3' position. The bulky 3-methoxymorpholino group is structurally distinct from an amino group, and therefore has a distinct and separate status in the art and is expected by those skilled in the art to have different properties (see structures below).

[1] The Examiner stated that Miura teaches the combination of doxorubicin and lipiodol. This is incorrect.



Doxorubicin



MMDX

Furthermore, because these two groups, and therefore the two compounds, are structurally distinct, there is no reason for a medicinal chemist to expect that the compounds would have the same biological activities, contrary to what might be predicted for homologs (e.g. methylamine v. ethylamine) or positional isomers (e.g. 2-chlorobenzene v. 3-chlorobenzene). For example, it is again noted that Kuhl teaches that both MMDX and doxorubicin have similar tumor specificity in CEM and K562 cells. In contrast, included with this response are several articles which suggest that doxorubicin and MMDX behave much differently in the enzymes DNA Topoisomerase I and II (Capranico, G. et al., *Molecular Pharmacology*, **45**(5): 908-915, 1994; Mariani, M., et al., *Invest. New Drugs*, **12**(2): 93-7, 1994; Wassermann, K., et al., van der Graaf, W.T., et al., *Cancer Chemother. Pharmacol.*, **35**: 345-48, 1995; Bielack, S.S., et al., *Anticancer Research*, **15**: 1279-1284, 1995; Duran, G.E., et al., Cancer *Chemother. Pharmacol.*, **38**: 210-216, 1996), i.e. doxorubicin having an effect on Topoisomerase II but not Topoisomerase I, and MMDX having an effect on Topoisomerase I but not Topoisomerase II. Clearly, the prior art demonstrates the uncertainty of predicting the activity of each molecule in any given model. The Examiner contends that "applicant has

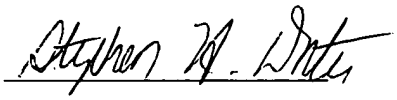
produced no evidence that the analog MMDX is so varied in its structure, effect and target that one of skill in the art would not have a reasonable expectation of success." Applicants now submit that, indeed, one of skill in the art would *not* have had a reasonable expectation of success, based on the findings of the prior art, to treat human liver tumors via the intrahepatic administration of MMDX, simply because it was an analog of doxorubicin.

Secondly, none of the cited references teach nor remotely suggest the use of MMDX in solid tumors such as liver tumors. As stated in the previous response, the potential of MMDX in the prior art, is assessed only in liquid tumor models, i.e. in a panel of 14 different human leukemia and lymphoma cell lines. While solid tumors are those that occur in organs, such as the liver, breast or the lung, liquid tumors consist of blood cells that have become cancerous, and the two types of tumors have a distinct etiology. In fact, Applicants include herewith references that teach doxorubicin and epirubicin, another anthracycline analog, have poor efficacy in treating liver cancer, or, in other words, teach *away* from the subject matter of the present claims (Lai, et al., *Cancer*, **62**(3): 479-83, 1988, Abstract included; Shepard, et al., *Reg. Cancer Treat.*, **3**(4): 197-201, 1990, Abstract included; Colleoni, et al., *Ann. Oncol.* (Meeting Abstract), **5**(Supp. 8), 1994; Ono, et al., *Semin. Oncol.*, **24**(2 Supp. 6): S6-18-S6-25, 1997, Abstract included; Lai, et al., *Arch. Surg.*, **133**(2): 183-8, 1998). A person skilled in the art would therefore have not been motivated to use another anthracyclin derivative such as MMDX, in light of the teachings of the prior art, with the reasonable expectation of obtaining a beneficial effect for the treatment of hepatic cancer. In contrast, Applicants have shown, through much experimentation, that MMDX chemotherapy through the hepatic artery is

effective for patients with solid liver tumors (see "Activity" section at page 13 of the specification). For the above reasons, withdrawal of the obviousness rejection is respectfully solicited.

Allowance of the claims and passage of the case to issue are respectfully solicited. Should the Examiner believe a discussion of this matter would be helpful, he is invited to telephone the undersigned at (312)-913-0001.

Respectfully submitted,

By: 

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References cited under item 1.

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MOLECULAR PHARMACOLOGY, 45:908-915

Influence of Structural Modifications at the 3′ and 4′ Positions of Doxorubicin on the Drug Ability to Trap Topoisomerase II and to Overcome Multidrug Resistance

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SUMMARY

To better define the role of the amino sugar in the pharmacological and biochemical properties of anthracyclines related to doxorubicin and daunorubicin, we have investigated the effects of various substituents at the 3′- and 4′-positions of the drug on cytotoxic activity and ability to stimulate DNA cleavage mediated by DNA topoisomerase II. The study shows that the nature of the substituent at the 3′-position but not the 4′-position is critical for drug ability to form cleavable complexes. The amino group at the 3′-position is not essential for cytotoxic and topoisomerase II-targeting activities, because it can be replaced by a hydroxyl group without reduction of activity. However, the presence of bulky substituents at this position (i.e., morpholinyl derivatives) totally inhibited the effects on the enzyme, thus supporting previous observations indicating that the cytotoxic potencies of these particular derivatives are not related to topoisomerase II inhibition. This conclusion is also supported by the observation that 3′-morpholinyl and 3′-methoxymorpholinyl derivatives are able to overcome atypical (i.e., topoisomerase II-mediated) multidrug resistance. Because a bulky substituent at the 4′-position did not reduce the ability to stimulate DNA cleavage, these results support a critical role of the 3′-position in the drug interaction with topoisomerase II in the ternary complex. An analysis of patterns of cross-resistance to the studied derivatives in resistant human tumor cell lines expressing different resistance mechanisms indicated that chemical modifications at the 3′-position of the sugar may have a relevant influence on the ability of the drugs to overcome specific mechanisms of resistance.

Like other intercalating agents with antitumor activity, anthracyclines exert their cytotoxic activity by interfering with DNA topoisomerase II function. Despite their apparent structural diversity, their molecular effects have been related to their ability to interfere with the breakage-rejoining action of topoisomerase II. Although the topoisomerase II inhibitors have a common intracellular target, the molecular basis of their variable therapeutic efficacy is still unknown. Similarly to other highly effective antitumor drugs, DOX stabilizes a transient DNA-topoisomerase II complex in which DNA strands are cut and covalently linked to the enzyme subunits (1–4). Investigations on the sequence specificity of DOX stimulation of *in vitro* DNA cleavage have led to a molecular model for drug action on topoisomerase II; drug molecules may be placed at the interface between the DNA cleavage site and the active site of the enzyme, thus forming DNA-drug-enzyme ternary complexes (5, 6).

The efficacy of DOX as an antitumor agent has stimulated many studies aimed at identifying critical substituents required for optimal activity. Previous studies on structure-activity relationships of anthracyclines have shown an important role for the structure and stereochemistry of the amino sugar (daunosamine) in the pharmacological and biochemical activity of anthracyclines related to DNR and DOX (7–10). The basic amino group at C-3′ has been implicated in determining the DNA binding affinity. However, the presence of a basic group at C-3′ is not a strict requirement for cytotoxic activity of anthracyclines (11). The role of substituents at the 3′-position remains unclear, because *N*-acyl derivatives exhibited low affinity for DNA and markedly reduced cytotoxic potency (7, 12), but substitution of the amino group for an hydroxyl group at C-3′ resulted in comparable cytotoxic activity (11).

The influence of selected chemical modifications at different

This work was partially supported by Consiglio Nazionale delle Ricerche and by Associazione Italiana per la Ricerca sul Cancro.

ABBREVIATIONS: DOX, doxorubicin; FCS, fetal calf serum; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; VM-26, 4′-demethylepipodophyllotoxin thenylidene-β-D-glucoside; SDS, sodium dodecyl sulfate; SV40, simian virus 40; MRP, multidrug resistance-related protein; DNR, daunorubicin; 4′-I-DOX, 4′-deoxy-4′-iododoxorubicin; ID_{50}, drug concentration inhibiting cell growth by 50%, compared with drug-free cultured cells; SCLC, small-cell lung cancer.

positions in the amino sugar has been examined with respect to the ability of the drug to trap DNA topoisomerase II (4, 13). In an attempt to better define the molecular pharmacology of anthracyclines, the present study was undertaken to examine the influence of various substituents at the 3′- and 4′-positions of DOX and/or DNR on the ability of the drugs to stimulate enzyme-mediated DNA cleavage. We provide evidence that (a) the 3′-position but not the 4′-position is critical for the ability of the drugs to interfere with topoisomerase II and (b) 3′-deamino-3′-hydroxy derivatives of DOX overcome *mdr1*-mediated but not atypical multidrug resistance.

Experimental Procedures

Materials. Anthracycline derivatives were synthetized at the Chemistry Department of Farmitalia-Carlo Erba (Milan, Italy). Drugs were dissolved in dimethylsulfoxide or deionized water at 0.1 mM, stored at −20° for a few weeks, and diluted in deionized water immediately before use. DNA topoisomerase II was purified from murine leukemia P388 cell nuclei by published procedures (14, 15) and was stored at −20° in 20 mM KH_2PO_4, pH 7.0, 50% glycerol, 0.5 mM phenylmethylsulfonyl fluoride, 0.1 mM EDTA, 1 mM β-mercaptoethanol. SV40 DNA, T4 polynucleotide kinase, agarose, and polyacrylamide were purchased from Bethesda Research Laboratories (Basel, Switzerland). [γ-^{32}P] ATP was purchased from Amersham (Milan, Italy). Calf intestinal phosphatase and restriction endonucleases were purchased from New England Biolabs (Taunus, Germany). Human *mdr1* and murine β-actin probes were as described previously (16).

Sequencing analysis of DNA cleavage sites. SV40 DNA fragments were uniquely 5′-end-labeled as described previously (5, 13). Briefly, SV40 DNA was cut with the indicated enzyme, dephosphorylated, and ^{32}P-labeled with T4 kinase. Then, DNA was subjected to a second enzyme digestion to generate uniquely 5′-end-labeled fragments, which were separated by agarose gel electrophoresis and purified by electroelution and ethanol precipitation. DNA cleavage reactions were performed in 20 μl of 10 mM Tris·HCl, pH 7.5, 50 mM NaCl, 50 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM ATP, 15 μg/ml bovine serum albumin, with drugs, at 37° for 20 min. Topoisomerase II (106 units, about 200 ng of protein) was added in storage buffer (14). Reactions were stopped with SDS (1%) and proteinase K (0.1 mg/ml) and were incubated at 42° for 45 min. DNAs were then precipitated with ethanol, resuspended in 2.5 μl of 80% formamide, 10 mM NaOH, 1 mM EDTA, 0.1% xylene cyanol, 0.1% bromphenol blue, heated at 95° for 2 min, chilled on ice, and then loaded onto a 8% polyacrylamide denaturing gel. Gels were run at 70 W for 2 hr. Autoradiograms of dried gels were carried out using Amersham Hyperfilm.

Cell lines. POGB and POVD cell lines were obtained in our laboratory from lung tumor biopsies of two patients bearing SCLC. At the time of biopsies, patients had not been treated with chemotherapy or radiotherapy. After about 10 passages in RPMI 1640 medium supplemented with 10 nM hydrocortisone, 10 nM 17-β-estradiol, 30 nM sodium selenite, 5 mg/ml insulin, 100 mg/ml transferrin, and 5% FCS (Flow Laboratories), cells were adapted to grow in RPMI 1640 medium supplemented with 10% FCS (CM medium). POGB cells grew loosely attached to the flask and, when needed, they were detached through trypsinization or strong pipetting. POVD cells grew as floating clumps. After 10 passages in CM medium, cells were cultured in the presence of 1 ng/ml DOX; in subsequent passages, the DOX concentration was progressively increased up to 120 ng/ml for POGB cells and 100 ng/ml for POVD cells. Drug-selected cells were then tested for their sensitivity to DOX and were shown to have become resistant to this drug. The established resistant cell variants POGB/DX and POVD/DX were maintained always in the presence of 120 or 100 ng/ml DOX, respectively. One passage before each experiment, resistant cells were cultured in drug-free medium. The morphologies of resistant cells were similar to those of sensitive parental cells. The human leukemic cell line CEM and the vinblastine- and VM-26-resistant sublines CEM/VLB_{100} and CEM/VM1 (17–19) were kindly provided by Dr. W. T. Beck (St. Jude Children's Research Hospital, Memphis, TN). The cell lines were maintained at 37° in minimal essential medium (GIBCO) with Earle's salts, supplemented with 10% FCS and 1% vitamins (GIBCO), and were passaged twice weekly. Vinblastine (100 ng/ml) or VM-26 (66 ng/ml) was added at each passage to CEM/VLB_{100} and CEM/VM1 cells, respectively. Parent and resistant CEM cells grew as floating clumps.

Cytotoxicity test. SCLC cells (10^5/ml) were treated for 1 hr at 37° with drugs at different concentrations. Cells were then washed with phosphate-buffered saline and seeded in drug-free CM medium in 96-well tissue culture plates (10^4 POGB cells/well and 5×10^3 POVD cells/well). Ninety-six hours later cell survival was determined with the MTT assay, as described previously (20).

Immediately after seeding, CEM, CEM/VLB_{100}, and CEM/VM1 cells (2×10^4 cells/ml) were treated for 72 hr at 37° with drugs at different concentrations. Cell survival was then determined by cell counting. IC_{50} values were determined from the dose-effect curves.

Northern blot analysis. Total RNA was prepared by the LiCl-guanidine monothiocyanate method (21) from cells harvested in the logarithmic phase of growth. Total RNA (20 μg) was fractionated on a formaldehyde-containing 1% agarose gel and then transferred to a Hybond nylon membrane. The membrane was then irradiated with UV light, and prehybridization was performed for at least 4 hr at 42°, in 50% formamide, 5× standard saline citrate (i.e., 0.75 M NaCl, 75 mM Na citrate, pH 7.2), 0.2% SDS, 5× Denhardt's solution, 50 mM sodium phosphate, pH 7, 250 μg/ml salmon sperm DNA. DNA probes were ^{32}P-labeled with a random primer kit (specific activity, $2–5 \times 10^8$ cpm/μg of DNA). Hybridization was carried out for 20 hr at 42° in the same buffer containing 10% dextran sulfate.

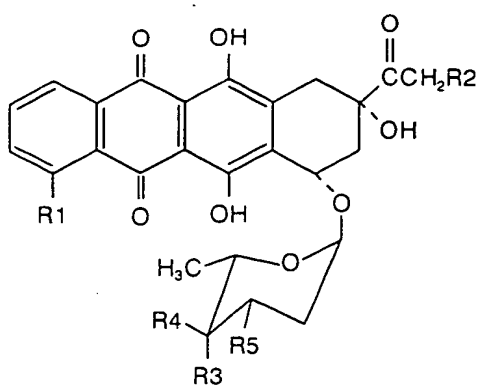
DNA binding studies. DNA binding parameters of anthracycline derivatives were determined by means of the fluorescence quencing method, as described previously (8–10), at the same ionic strength (0.1 M NaCl).

Results

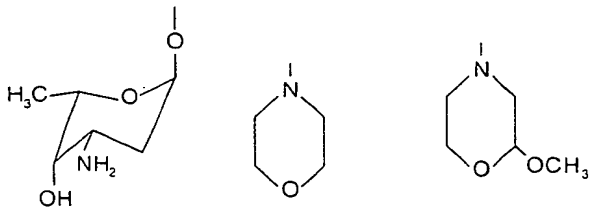
Stimulation of topoisomerase II DNA cleavage. The effect of various substituents at the 3′- and 4′-positions of the anthracycline molecule (Fig. 1) on the stimulation activity of topoisomerase II DNA cleavage was investigated with the experiments shown in Figs. 2 and 3. Drug stimulation of DNA cleavage was determined by incubating 5′-^{32}P-labeled SV40 DNA fragments with topoisomerase II and different concentrations of the analogs and analyzing DNA cleavage intensity patterns with polyacrylamide denaturing gels. Drug analogs were always compared with either DOX or 4-demethoxy-DNR, which have been shown to stimulate identical DNA cleavage intensity patterns (5).

3′-Morpholinyl and 3′-methoxymorpholinyl analogs of DOX did not stimulate enzyme-mediated DNA cleavage; however, they suppressed DNA cleavage at 1 and 10 μM (see 4880 site in Fig. 2). In contrast, 3′-deamino-3′-hydroxy-4′-morpholinyl-DOX stimulated DNA cleavage at the same sites and with similar relative intensities, compared with the parent drug (Fig. 2). Because the 3′-deamino-3′-hydroxy-4′-morpholinyl analog stimulated topoisomerase II DNA cleavage, the chemical nature of the morpholinyl group *per se* may not explain the inability of the 3′-morpholinyl derivatives to interfere with topoisomerase II function. Thus, the relative position of the substitution (3′ versus 4′) appeared to be the critical structural feature of the drug for retention of stimulation activity of DNA cleavage.

We then investigated the 3′-deamino-3′-hydroxy derivatives of DOX shown in Fig. 1. 3′-Deamino-3′-hydroxy-4′-amino-DOX and 3′-deamino-3′-hydroxy-4′-epi-DOX stimulated DNA cleavage to similar extents, compared with the parent



Compound	R1	R2	R3	R4	R5
Doxorubicin (DOX)	OCH_3	OH	OH	H	NH_2
Daunorubicin (DNR)	OCH_3	H	OH	H	NH_2
3'deamino-3'hydroxy -4'epiDOX	OCH_3	OH	H	OH	OH
4demethoxy-3'deamino-3'hydroxy-4'epiDOX	H	OH	H	OH	OH
3'deamino-3'hydroxy-4'aminoDOX	OCH_3	OH	NH_2	H	OH
3'morpholinylDOX	OCH_3	OH	OH	H	(b)
3'methoxy morpholinylDOX	OCH_3	OH	OH	H	(c)
3'deamino-3'hydroxy-4'morpholinylDOX	OCH_3	OH	(b)	H	OH
4'daunosaminylDNR	OCH_3	H	(a)	H	NH_2
4'I-DOX	OCH_3	OH	I	H	NH_2



(a) Daunosaminyl (b) Morpholinyl (c) Methoxymorpholinyl

Fig. 1. Chemical structures of the studied anthracycline derivatives.

drug (Fig. 2). 4-Demethoxy-3'-deamino-3'-hydroxy-4'-epi-DOX was the most potent analog in stimulating DNA cleavage, because it was effective even at 0.1 μM. Because 4'-epimerization has been shown to have no effect on cleavage stimulation by anthracyclines (13), these observations indicated that the amino group at the 3'-position is not necessary for the drug effect on topoisomerase II. Indeed, lack of the amino group in the sugar increased the stimulation activity of anthracyclines for DNA cleavage (Fig. 2). 4'-Morpholinyl-DOX, 3'-deamino-3'-hydroxy-4'-epi-DOX, and 4-demethoxy-3'-deamino-3'-hydroxy-4'-epi-DOX even at 10 μM stimulated DNA cleavage, whereas global suppression of DNA cleavage was observed at 10 μM for DOX, 3'-morpholinyl analogs, and 3'-deamino-3'-hydroxy-4'-amino-DOX (Fig. 2 and data not shown; see also Ref. 13). A lack of suppressive effect of the 3'-hydroxy derivatives may be rationalized in terms of reduced DNA binding affinity (Table 1), causing a different biphasic response.

To further evaluate the role of the 4'-position, we investigated 4'-*O*-daunosaminyl-DNR and 4'-I-DOX, which have an additional amino sugar and an iodine atom, respectively, at the 4'-position (Fig. 1). Both of these analogs stimulated cleavage of SV40 DNA in the presence of topoisomerase II (Fig. 3). 4'-I-DOX has been shown to stimulate protein-associated DNA breaks in living tumor cells (22).

DNA cleavage intensity patterns were identical among all of the studied analogs (Figs. 2 and 3). Weak cleavage sites were, however, more easily detected with the most potent derivatives (see sites from 4779 to 4814 in Fig. 2). The sequence specificity of DNA cleavage stimulation by the studied derivatives was thus the same as that reported for the parent drugs (5).

Cytotoxic potency of the anthracycline derivatives in human SCLC cell lines. All of the studied analogs were cytotoxic against human SCLC cells, although differences in potency could be noted. 3'-Deamino-3'-hydroxy-4'-epi-DOX was 3–4-fold less potent than DOX, and 4-demethoxy-3'-deamino-3'-hydroxy-4'-epi-DOX was about as potent as the parent drug (Table 2). The increased potency of 4-demethoxy-3'-deamino-3'-hydroxy-4'-epi-DOX, compared with 3'-deamino-3'-hydroxy-4'-epi-DOX, showed that removing the methoxy group at the 4-position in the planar ring system enhanced drug cytotoxic activity, in agreement with previous findings (23, 24). Because 4'-epimerization did not affect DOX cytotoxicity (8), removal of the 3'-amino group appears to be responsible for the reduced cell-killing activity. No precise correlation was found between the cytotoxic potency and DNA-cleaving activity, because these analogs were more active than DOX in stimulating topoisomerase II DNA cleavage (Fig. 2). Therefore, it may be possible that cellular pharmacokinetics of these analogs were somewhat altered by the chemical modification at the 3'-position, compared with DOX.

3'-Deamino-3'-hydroxy-4'-amino-DOX and 4'-*O*-daunosaminyl-DNR were somewhat less potent than DOX in SCLC cell lines (Table 2). However, they have been shown to be 2–3 times more potent than and as potent as the parent drug, respectively, in a sensitive CEM human leukemia cell line (25).

3'-Morpholinyl analogs were 3–6-fold more potent than DOX in the two sensitive SCLC cell lines, POGB and POVD (Table 2), in agreement with previous findings showing marked cytotoxic activity of 3'-morpholinyl-anthracyclines (26–28). In contrast, 4'-morpholinyl-DOX was similar to DOX, or slightly less potent than the parent drug (Table 2). Consistently, 4'-morpholinyl-DOX was 2.5-fold less potent, whereas 3'-morpholinyl analogs were about 4.5-fold more potent than DOX in an unrelated SCLC cell line, NCI-H187 (data not shown).

Cell-killing activity of drugs in human multidrug-resistant tumor cell lines with different mechanisms of resistance. The cytotoxic activities of the studied derivatives were also determined in the multidrug-resistant variants of the POGB and POVD SCLC lines (Table 2) and in two multidrug-resistant lines derived from a CEM human leukemia line (16–18) (Table 3). Both POVD/DX and POGB/DX lines were obtained from the corresponding sensitive lines by DOX selection (see Experimental Procedures for details).[1] It is likely that different mechanisms of drug resistance have been activated in these two DOX-selected SCLC sublines. Overexpression of the *mdr1* gene was found in POVD/DX cells, compared with the

[1] M. Binaschi, R. Supino, G. Capranico, and F. Zunino. Multidrug resistance in small cell lung cancer cell lines. Manuscript in preparation.

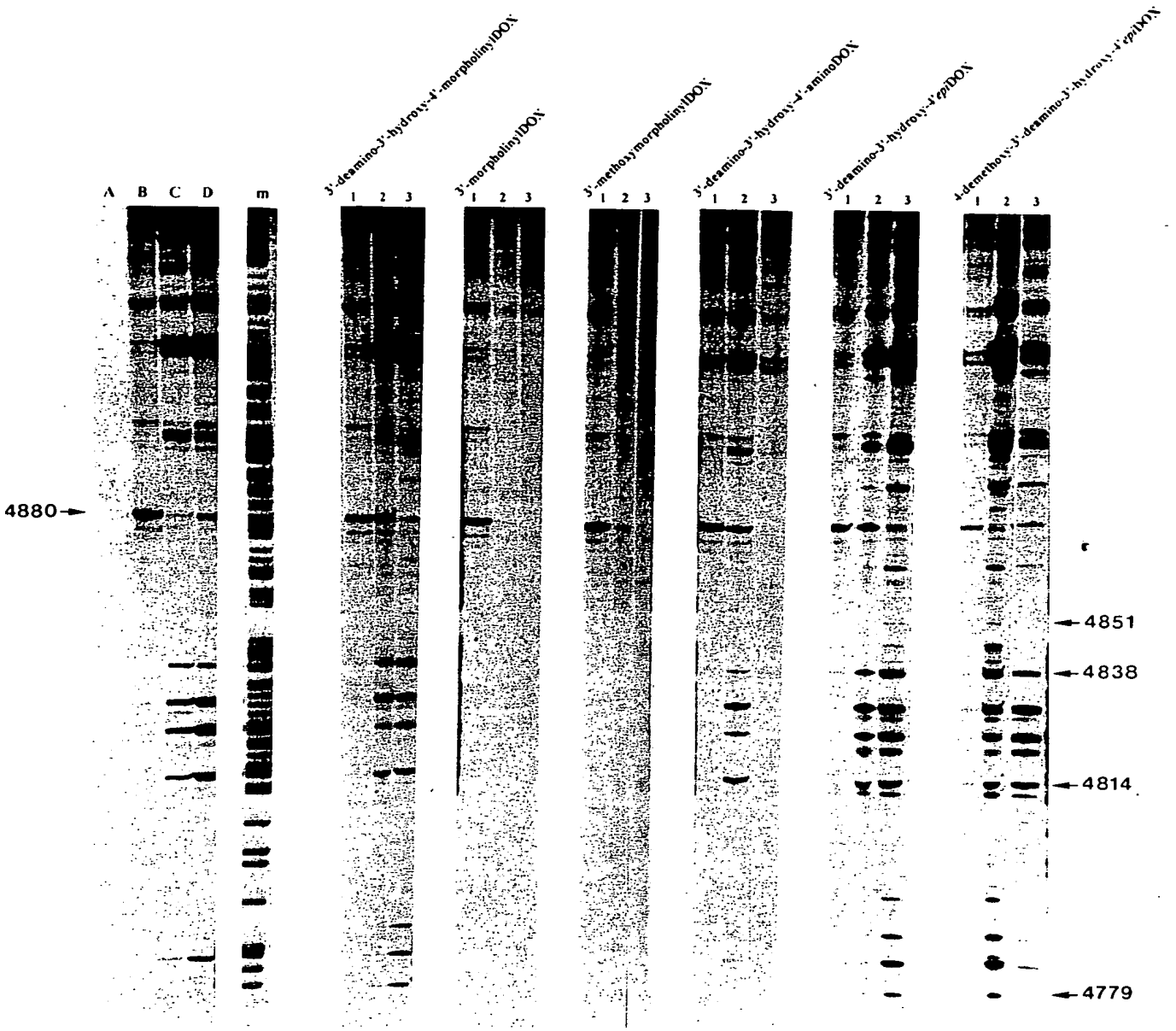


Fig. 2. Topoisomerase II DNA cleavage stimulated by the studied DOX derivatives in SV40 DNA. SV40 DNA ^{32}P-labeled at the *TaqI* site was incubated for 20 min at 37° with purified murine topoisomerase II and different concentrations of drugs. Cleavage reactions were stopped with 1% SDS and 0.1 mg/ml proteinase K, incubated at 42° for 45 min, precipitated with ethanol, and then analyzed on 8% polyacrylamide sequencing gels. *Lane A*, control DNA; *lane B*, topoisomerase II alone; *lane C*, with 1 μM DOX; *lane D*, with 1 μM 4-demethoxy-DNR; *lane m*, purine molecular weight markers; *lanes 1*, *2*, and *3*, 0.1, 1, and 10 μM indicated drug, respectively. *Arrows*, some cleavage sites; *numbers*, genomic position in SV40 DNA.

sensitive parental POVD cells (Fig. 4). In these experiments human colon cancer LoVo and LoVo/DX cells were used as a control system for P-glycoprotein overexpression (29). POVD/DX exhibited a pattern of cross-resistance typical of the multidrug-resistant phenotype, because it included vincristine, etoposide, and taxol but not cisplatin, melphalan, or 5-fluorouracil. In contrast, *mdr1* gene expression was not detected in POGB/DX cells, which instead exhibited amplification (about 50-fold) and overexpression of the MRP gene.[2] The phenotypes of multidrug-resistant CEM sublines have been shown to be distinct (17–19). CEM/VLB$_{100}$ cells had a classical multidrug-resistant phenotype and overexpressed the *mdr1* gene, whereas CEM/VM1 cells showed an atypical multidrug-resistant phenotype and did not overexpress the *mdr1* gene but instead had a mutated topoisomerase IIα gene coding for a drug-resistant protein (17–19).

The studied 3′-deamino-3′-hydroxy-4′-epi derivatives could overcome the drug resistance of POGB/DX, POVD/DX, and CEM/VLB cells but not that of CEM/VM1 cells (Tables 2 and 3). These observations suggested that an amino group in the sugar moiety of DOX might be critical for drug transport by P-glycoprotein. 3′-Morpholinyl derivatives were as active in all of the drug-resistant variant lines as in the corresponding

[2] M. Binaschi, R. Supino, R. A. Gambetta, G. Giaccone, E. Prosperi, G. Capranico, and F. Zunino. MRP gene overexpression and amplification in a human doxorubicin-resistant SCLC cell line.

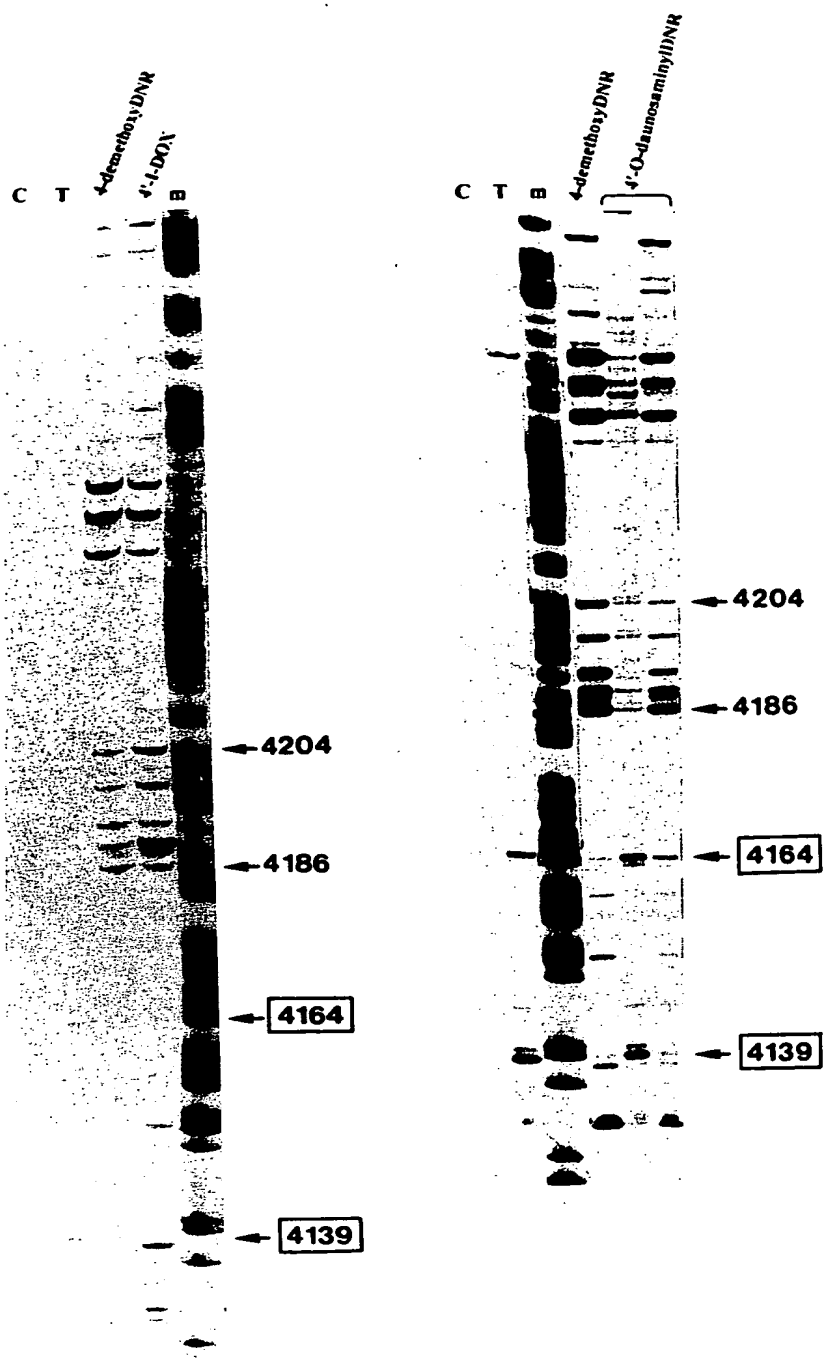


Fig. 3. Topoisomerase II DNA cleavage stimulated by the studied DOX derivatives in SV40 DNA. SV40 DNA was ^{32}P-labeled at a *Xho*II site. See legend to Fig. 2 for additional details. *Lane C*, control DNA; *lane T*, topoisomerase II alone; *lane m*, purine molecular markers. 4-Demethoxy-DNR and 4′-I-DOX were used at 1 μM. 4′-*O*-Daunosaminyl-DNR was used at 0.1 and 1 μM in the *left* and *right lanes*, respectively. *Arrows*, cleavage sites; *numbers*, genomic position in SV40 DNA. *Boxed numbers*, sites not stimulated by drugs.

parent cell lines. In contrast, CEM/VM1 but not CEM/VLB cells were fully cross-resistant to 3′-deamino-3′-hydroxy-4′-morpholinyl-DOX, supporting the idea that this derivative was directed against topoisomerase II also in living tumor cells.

The patterns of cross-resistance of these variant lines were not similar (Tables 2 and 3). Some observations can be made. (a) 3′-Morpholinyl derivatives were the only drugs showing no cross-resistance in the CEM/VM1 cell line, whereas all other drugs showed cross-resistance at similar or higher levels, compared with DOX (Table 3). (b) Only 4′-*O*-daunosaminyl-DNR and 3′-deamino-3′-hydroxy-4′-amino-DOX showed cross-resistance fully in CEM/VLB cells and at the highest level among the studied drugs in POVD/DX cells (Tables 2 and 3). Both of these analogs retained an amino group in the sugar moiety, and these two lines were drug resistant due to overexpression of the P-glycoprotein gene. (c) POGB/DX cells showed a specific pattern of cross-resistance; these cells were cross-resistant only to 3′-deamino-3′-hydroxy-4′-morpholinyl-DOX and 4′-*O*-daunosaminyl-DNR. This finding was consistent with a different mechanism of resistance developed by POGB/DX cells.

Discussion

Previous studies from this laboratory demonstrated the importance of the natural amino sugar (daunosamine) as a critical

TABLE 1

Binding parameters for the interaction of anthracycline derivatives with calf thymus DNA

K_{app} is the apparent binding constant; n is the apparent binding sites/nucleotide.

	K_{app} ($\times 10^6$)	n
	M^{-1}	
DNR	4.8	0.160
DOX	6.5	0.179
3′-Deamino-3′-hydroxy-4′epi-DOX	0.77	0.062
4-Demethoxy-3′-deamino-3′-hydroxy-4′epi-DOX	1.0	0.072
3′-Deamino-3′-hydroxy-4′amino-DOX	2.4	0.178
4′-I-DOX	6.4	0.100
4′-*O*-Daunosaminyl-DNR	2.7	0.283

determinant of anthracycline activity (8). In agreement with the view that DNA topoisomerase II is the primary target of drug action (4), we now provide evidence that the sugar moiety is also important for drug stimulation of topoisomerase II-mediated DNA cleavage. The 3′-*N*-substituted anthracyclines exhibited a reduced ability to stimulate topoisomerase II-mediated cleavage (30). The inability of 3′-morpholinyl and 3′-methoxymorpholinyl, but not 4′-morpholinyl, derivatives of DOX to stimulate enzyme-mediated DNA cleavage is consistent with these observations (see also Ref. 31) and suggests that a bulky substituent at the 3′-position is a steric hindrance for the formation of the ternary complex (drug-enzyme-DNA). Previous studies have emphasized the role of the amino group in the stabilization of the intercalation complex (32), and a free protonated amino group has been implicated in electrostatic interactions in the minor groove (33–35). Indeed, *N*-acetyl derivatives of DNR and DOX exhibited low DNA binding activity and markedly reduced cytotoxic and antitumor potencies. In contrast, because 3′-morpholinyl and 3′-methoxymorpholinyl derivatives are still very potent cytotoxic agents, it is evident that these compounds may exert cytotoxic activity by other mechanisms that are independent of topoisomerase II inhibition. 3′-Methoxymorpholinyl-DOX has a potential for covalent binding to DNA (36), and the formation of a drug-DNA adduct by active 3′-morpholinyl-DOX metabolites has been reported (27). 3′-Morpholinyl-DOX itself was found to retain DNA-binding ability and to stimulate topoisomerase I-induced DNA cleavage (31). Further investigations at a cellular level are required to better understand the mechanism of action of these derivatives.

The present study provides further evidence that an amino group at the 3′-position is not required to stimulate topoisomerase II-mediated DNA cleavage, because substitution of a hydroxyl group for the amino group resulted in compounds (i.e., 3′-deamino-3′-hydroxy-4′-epi-DOX and 4-demethoxy-3′-deamino-3′-hydroxy-4′-epi-DOX) with activity comparable or superior to that of the corresponding 3′-aminoanthracyclines. This finding is consistent with previous observations that 3′-hydroxy derivatives retained cytotoxic and antitumor activities (11, 25). In contrast to 3′-morpholinyl-DOX, the compound with this substituent at the 4′-position has been found to have the same ability to trap topoisomerase II as exhibited by the parent compound, DOX. Similarly, different substitutions at the 4′-position (i.e., 4′-*O*-daunosaminyl-DNR and 4′-I-DOX) resulted in agents effective as topoisomerase II inhibitors. Overall, these results indicated that the presence of a bulky substituent at the 3′- but not the 4′-position prevents drug stimulation of topoisomerase II cleavage. As expected, the removal of the amino group in the sugar moiety caused an appreciable reduction of drug affinity for DNA. However, this reduction was accompanied by an appreciable increase in drug ability to trap DNA topoisomerase. A lack of precise correlation between the DNA binding affinity and the cellular and molecular effects of anthracyclines supports the view that the specific mode of DNA interaction is a more critical determinant for drug activity than is the strength of binding (4).

All of the tested 3′-hydroxy derivatives (Table 2) showed marked activity in the cytotoxicity assay. Using SCLC cell lines, the pattern of cross-resistance indicated that, with the exception of 3′-deamino-3′-hydroxy-4′-amino-DOX, all 3′-hydroxy derivatives overcame multidrug-resistance mediated by *mdr1* gene overexpression (i.e., POVD/DX cell line). It is possible that the presence of a free amino group is an important determinant for drug recognition by P-glycoprotein, because 4′-*O*-daunosaminyl-DNR also displays partial cross-resistance in this cell system. Alternatively, by removal of the sugar amino group the drug becomes more lipophilic, and this may favor cellular drug uptake by passive diffusion, thus counteracting the P-glycoprotein-dependent increased drug efflux in resistant cells. Similar results were obtained in the CEM/VLB cell line, with a typical multidrug resistant phenotype (Table 3).

The pattern of cross-resistance was somewhat different in the POGB/DX cell line. In this system, only compounds with a bulky substituent at the 4′-position showed cross-resistance. This subline exhibited a multidrug resistant phenotype with reduced intracellular drug accumulation, without *mdr1* expression.[2] It is possible that other transport systems (MRP gene

TABLE 2

Cytotoxic activities and cross-resistance of anthracycline derivatives in human SCLC cell lines

POVD/DX cells showed a classical multidrug-resistant phenotype and overexpressed the *mdr1* gene (Fig. 4); POGB/DX cells did not express the *mdr1* gene but had amplification and overexpression of the MRP gene.[2] IC_{50} values were determined from dose-response curves after 1-hr exposure to the drug. The shapes of the dose-response curves were similar for all tested compounds. In parentheses is the resistance index, calculated as the ratio of the IC_{50} values in DOX-resistant and parental cell lines.

Drug	IC_{50}			
	POGB	POGB/DX	POVD	POVD/DX
	$\mu g/ml$			
DOX	0.30	1.90 (6.3)	0.30	4.80 (16)
3′-Deamino-3′-Hydroxy-4′epi-DOX	1.22	2.00 (1.6)	1.00	2.10 (2.1)
4-Demethoxy-3′-deamino-3′-hydroxy-4′epi-DOX	0.22	0.25 (1.1)	0.41	0.25 (0.6)
3′-Deamino-3′-hydroxy-4′-amino-DOX	0.80	1.30 (1.6)	0.62	3.20 (5.2)
3′-Morpholinyl-DOX	0.12	0.14 (1.2)	0.05	0.17 (3.4)
3′-Methoxymorpholinyl-DOX	0.10	0.18 (1.8)	0.05	0.06 (1.2)
3′-Deamino-3′-hydroxy-4′-morpholinyl-DOX	0.38	1.55 (4.1)	0.70	1.20 (1.7)
4′-*O*-Daunosaminyl-DNR	0.88	3.95 (4.5)	0.76	3.65 (4.8)

TABLE 3

Cross-resistance to the studied anthracycline derivatives of two human multidrug-resistant CEM cell lines

CEM/VLB$_{100}$ cells showed a classical multidrug-resistant phenotype and overexpressed the *mdr1* gene, whereas CEM/VM1 cells showed an atypical multidrug-resistant phenotype and had a mutated topoisomerase IIα gene (16-18). Drug treatments were for 72 hr at 37°; drugs were then washed out, and cell survival was evaluated by cell counting. ID_{50} values were 3.3, 72.3, and 28.7 ng/ml for 3′-deamino-3′-hydroxy-4′-amino-DOX and 6.1, 270, and 15.5 ng/ml for 4′-daunosaminyl-DNR in the parent CEM, CEM/VLB, and CEM/VM1 cell lines, respectively. ID_{50} values for the other drugs were reported previously (24).

Drug	Resistance index relative to sensitive CEM line	
	CEM/VLB	CEM/VM1
DOX	31	5.7
4-Demethoxy-DNR	2.8	4.8
3′-Deamino-3′-hydroxy-4′epi-DOX	3.9	13.2
4-Demethoxy-3′-deamino-3′-hydroxy-4′epi-DOX	0.9	8.1
3′-Deamino-3′-hydroxy-4′-amino-DOX	22	8.7
3′-Morpholinyl-DOX	2.4	2.0
3′-Methoxymorpholinyl-DOX	1.0	1.1
3′-Deamino-3′-hydroxy-4′-morpholinyl-DOX	1.2	5.6
4′-O-Daunosaminyl-DNR	44	2.5
4′-I-DOX	1.2	2.9

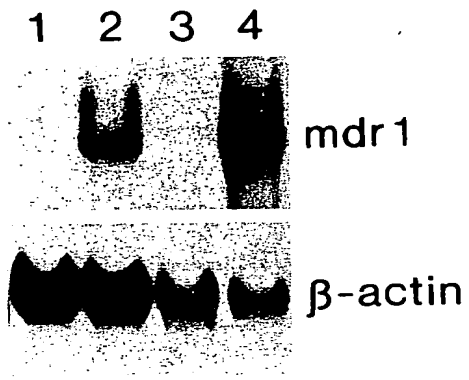


Fig. 4. Overexpression of the *mdr1* gene in DOX-resistant POVD/DX cells. Twenty micrograms of total RNA were fractionated on a 1% agarose-formaldehyde gel, transferred to a nylon membrane, and hybridized with the indicated human probes. *Lane 1*, POVD; *lane 2*, POVD/DX; *lane 3*, LoVo; *lane 4*, LoVo/DX.

product?) are involved, conferring a different cross-resistance pattern. Among the compounds examined, only 3′-morpholinyl and 3′-methoxymorpholinyl derivatives were found to be able to overcome resistance mediated by topoisomerase II gene mutations (i.e., in CEM/VM1 cells). This finding is in agreement with the hypothesis that these derivatives differ from conventional anthracyclines, with the natural amino sugar, in their mechanism of action (27). In CEM/VM1 cells all 3′-hydroxy derivatives were found to be cross-resistant, with a resistance index comparable or superior to that of DOX. Again, this observation is consistent with the conclusion that 3′-hydroxy derivatives exert cytotoxic activity through inhibition of topoisomerase II function. The inability of these compounds to overcome topoisomerase II-mediated resistance is expected on the basis of the identical sequence specificities of DNA cleavage stimulation, compared with DOX (5), suggesting a similar structural basis of drug interaction with topoisomerase II in the ternary complex.

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Growth-inhibitory properties of novel anthracyclines in human leukemic cell lines expressing either Pgp-MDR or at-MDR.

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The objective of the experiments reported in this paper was the identification of promising anthracycline analogs on the basis of lack of cross-resistance against tumor cells presenting either P-glycoprotein multidrug resistance (Pgp-MDR) or the altered topoisomerase multidrug resistant (at-MDR) phenotype. Differently modified anthracycline analogs known to be active against MDR cells were assayed in vitro against CEM human leukemic cells, and the sublines CEM/VLB100 and CEM/VM-1 exhibiting respectively the Pgp-MDR and the at-MDR phenotype. Two classes of molecules, in which the -NH2 group in C-3' position is substituted with a morpholino, methoxymorpholino (morpholinyl-anthracycline), or an alkylating moiety, present equivalent efficacy in the drug-sensitive and the two drug-resistant sublines. These results indicate that such molecules may exert their cytotoxic effect through a mode of action different from that of "classical" anthracyclines and is not mediated through topoisomerase II inhibition. Both molecules represent novel concepts in the field of new anthracyclines derivatives.

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SHORT COMMUNICATION

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The role of methoxymorpholino anthracycline and cyanomorpholino anthracycline in a sensitive small-cell lung-cancer cell line and its multidrug-resistant but *P*-glycoprotein-negative and cisplatin-resistant counterparts

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stract The cytotoxic action of two morpholino anthracyclines, methoxymorpholino anthracycline (MRA-MT, FCE 23762) and cyanomorpholino anthracycline (MRA-CN), was compared with the cytotoxicity of doxorubicin (DOX), the topoisomerase II inhibitor etoposide (VP-16), the topoisomerase I inhibitor camptothecin, methotrexate, and cisplatin in GLC4, a human small-cell lung-cancer cell line, in GLC4-Adr, its P-glycoprotein (Pgp)-negative, multidrug-resistant (MDR; 100-fold DOX-resistant) subline with overexpression of the MDR-associated protein (MRP) and a lowered topoisomerase II activity, and in GLC4-CDDP, its cisplatin-resistant subline. GLC4-Adr was about 2-fold cross-resistant for the morpholino anthracyclines and GLC4-CDDP was, relative to GLC4, more resistant for the morpholino anthracyclines than for DOX. Overall, MRA-CN was about 2.5-fold more cytotoxic than MRA-MT. The cytotoxicity profile of the morpholino anthracyclines in these cell lines mimicked that of camptothecin.

y words Morpholino anthracyclines · MRP
isplatin resistance

Introduction

The development of multidrug resistance (MDR) is one of the major obstacles in successful chemotherapeutic treatment of cancer patients. One class of antitumor drugs with the widest spectrum of activity in human cancers comprises the anthracyclines, which, however, are involved in MDR [1]. For doxorubicin (DOX), one of the most frequently used drugs of this group of anthracyclines, several mechanisms contribute to its cytotoxic action. Cellular mechanisms of action include intercalation of DNA; the formation of DNA breaks, possibly due to the generation of free radicals; and the capacity to turn DOX-topoisomerase complexes into cellular poisons [2, 3]. Because of its toxicity, especially cardiac toxicity, and the appearance of drug resistance, new anthracycline analogs have been synthesized that are less toxic, more potent, and non-cross-resistant with DOX. One group of these analogs consists of compounds in which a morpholino ring incorporating the amino nitrogen of the daunosamine unit has been constructed. Acton et al. [4] synthesized a series of morpholino (e. g., MRA) and cyanomorpholino (e. g., MRA-CN) analogs of DOX. These morpholinyl-substituted anthracyclines have several properties in common that distinguish them from the parental anthracyclines. They are highly lipophilic, which facilitates rapid diffusion through the cell membrane [4, 5]. In contrast to DOX, MRA and MRA-CN are not cardiotoxic at effective antitumor doses [4, 6] and are not cross-resistant in DOX-resistant P-glycoprotein (Pgp)-positive and -negative cell lines [6–9]. Apart from its efficacy in Pgp-positive cell lines resistant to DOX, MRA-MT has also been proven effective in CEM/VM-1, a cell line with altered topoisomerase II, and in cell lines resistant to cisplatin and melphalan [10–13].

The working mechanisms of MRA and MRA-CN include preferential inhibition of ribosomal gene transcription [14] as well as topoisomerase I-mediated DNA cleavage [15]. This mechanism of cytotoxicity is probably different from that of DOX, which acts on topoisomerase II. Whereas MRA binds to DNA by intercalation and causes DNA strand breaks, MRA-CN produces DNA-DNA interstrand cross-links [15, 16]. It has been demonstrated that this interstrand DNA cross-link formation induced by MRA-CN, which takes place very rapidly, is preceded by the binding of drug to single-stranded DNA [17]. The marked difference in cytotoxicity and DNA-binding affinity observed between MRA and MRA-CN suggests a major role for the cyano substituent in the action of MRA-CN [18].

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Table 1 ID_{50} values after 1 h incubation as determined in the MTA. Results are expressed as mean values (± SD) for 2–4 experiments performed in quadruplicate

	Cell lines		
	GLC4	GLC4-Adr	GLC4-CDDP
DOX (μM)	0.33 ±0.07	30.1 ±8.0	0.17 ±0.06
MRA-MT (μM)	0.0079±0.0017	0.020±0.005	0.030±0.009
MRA-CN (μM)	0.0045±0.0018	0.0052±0.0020	0.0088±0.0009

Table 2 ID_{50} values after continuous incubation as determined in the MTA. Results are expressed as mean values (± SD) for 2–7 experiments performed in quadruplicate

	Cell lines		
	GLC4	GLC4-Adr	GLC4-CDDP
DOX (nM)	32.5 ±2.1	3.732 ±336	42.3 ± 1.0
VP-16 (nM)	0.16±0.03	10.2 ± 2.4	0.11± 0.02
Camptothecin (nM)	6.1 ±2.5	7.6 ± 0.9	30.8 ±14.4
Methotrexate (μM)	0.05±0.03	0.14± 0.04	0.08± 0.02
Cisplatin (μM)	0.90±0.07	2.2 ± 0.2	11.9 ± 2.2
MRA-CN (nM)	0.59±0.16	1.4 ± 0.4	2.1 ± 1.4

In the present study the role of MRA-CN and MRA-MT in cell lines with well-defined, different patterns of resistance, namely, non-Pgp MDR and cisplatin resistance, was tested and compared with the cytotoxicity of these compounds in a sensitive cell line.

Materials and methods

GLC4 is a Pgp-negative human small-cell carcinoma cell line [19] and GLC4-Adr is the DOX-resistant subline of GLC4. It shows an atypical MDR phenotype with resistance to DOX, vincristine, VP-16, and m-AMSA without *mdr*-1 gene amplification or Pgp expression [20]. In GLC4-Adr, a membrane efflux pump different from Pgp and overexpression of a mainly cytoplasmic 110-kDa protein detectable with the monoclonal antibody LRP-56 as well as overexpression of the new putative membrane transporter gene MRP were demonstrated [21–23]. GLC4-CDDP is the cisplatin-resistant subline of GLC4 with a 13.2-fold resistance to cisplatin due to increased glutathione (GSH), unchanged glutathione S-transferase (GST), decreased DNA platination, and increased repair of platinum adducts [24, 25]. Topoisomerase II activity proved to be 100% in GLC4, 35% in GLC4-Adr, and 130% in GLC4-CDDP [26]. Topoisomerase I activity did not differ among these cell lines [20]. GSH levels are 2.5-fold higher in GLC4-CDDP and 2.1-fold higher in GLC4-Adr as compared with GLC4; GST activity is equal in GLC4 and GLC4-CDDP but is 1.7-fold higher in GLC4-Adr [25, 27]. The doubling times of these cell lines are as follows: GLC4, 16.5 h; GLC4-Adr, 21.8 h; and GLC4-CDDP, 28.0 h [25, 28]. All cell lines were cultured in RPMI 1640 medium and 10% fetal calf serum (FCS) at 37 °C in a humidified atmosphere containing 5% CO_2.

A drug-sensitivity assay was performed with the microculture tetrazolium assay (MTA) as described previously [28]. To assure linearity the following numbers of cells per well (0.1 ml) were incubated: GLC4, 5,000; GLC4-Adr, 12,500; and GLC4-CDDP, 15,000. Cells were incubated with chemotherapeutic drugs either continuously for 4 days or for 1 h. When incubated for 1 h, the cells were washed. All assays were performed two to seven times in quadruplicate. The results are expressed as the mean (± SD) doses required to inhibit the growth of each cell line by 50% (ID_{50} values).

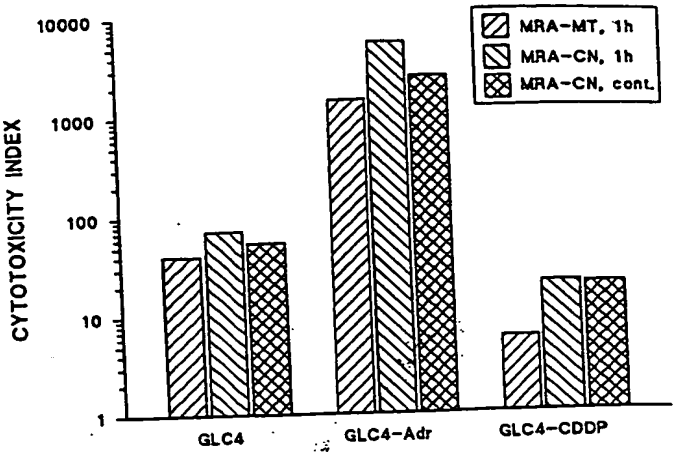

Fig. 1 Cytotoxicity index (ratio of the ID_{50} for DOX versus the ID_{50} for morpholino anthracyclines as determined in the MTA) obtained for each cell line

To measure the effect of buthionine sulfoximine (BSO) pretreatment on MRA-CN-induced cytotoxicity, GLC4, GLC4-Adr, and GLC4-CDDP cells were cultured for 48, 24, and 48 h, respectively, in the presence of 50 μM BSO without growth delay or loss of viability. Subsequently, MRA-CN-induced cytotoxicity (continuous incubation) was measured in the MTA (n = 3–4 experiments performed in quadruplicate).

Results

Tables 1 and 2 show the results of the 1-h and continuous incubations, respectively, in the MTA. Both morpholino anthracyclines are much more potent than DOX in these cell lines. The cytotoxicity of the morpholinyl derivates is remarkable in the highly DOX-resistant GLC4-Adr line. In contrast, GLC4-CDDP is relatively more sensitive to DOX but less sensitive to MRA-MT and MRA-CN than is GLC4. This comparison of the cytotoxicity sensitivity of the different cell lines for DOX, MRA-MT, and MRA-CN is expressed in Fig. 1. In this figure the cytotoxicity index is shown, which represents the ratio of the ID_{50} determined for DOX either after a 1-h incubation or after continuous incubation in a certain cell line versus the ID_{50} found for MRA-MT or MRA-CN. This demonstrates the potency of MRA-MT and MRA-CN with respect to DOX but also gives an impression about the mutual efficacy of the two morpholino anthracyclines and about the possibly different results of short versus continuous incubation. In all cell lines the cytotoxic action of the morpholino compounds surpassed the cytotoxicity of DOX. For both MRA-MT and MRA-CN the cytotoxicity indices were lowest in GLC4-CDDP and highest in GLC4-Adr. In the 1-h incubations, MRA-CN was 1.8-fold more active than MRA-MT in GLC4, 3.4-fold more active in GLC4-CDDP, and 3.8-fold more active in GLC4-Adr. In GLC4 and GLC4-Adr, 1-h incubations of MRA-CN seemed more effective than continuous incubations, whereas in GLC4-CDDP there was no difference.

Table 3 Cross-resistance factors[a] of GLC4-Adr and GLC4-CDDP versus GLC4 for DOX, MRA-MT, and MRA-CN

	Cell lines	
	GLC4-Adr	GLC4-CDDP
DOX	91	0.5
MRA-MT	2.5	3.8
MRA-CN	1.2	2.0

[a] At the ID_{50} as determined in the MTA (1 h incubation)

Table 4 Cross-resistance factors[a] of GLC4-Adr and GLC4-CDDP versus GLC4 for DOX, VP-16, camptothecin, methotrexate, cisplatin and MRA-CN

	Cell lines	
	GLC4-Adr	GLC4-CDDP
DOX	115	1.3
VP-16	64	0.7
nptothecin	1.2	5.0
Methotrexate	2.8	1.6
Cisplatin	2.3	13.2
MRA-CN	2.4	3.6

[a] At the ID_{50} as determined in the MTA (continuous incubation)

The results of the MTA also led to cross-resistance factors, which are shown in Tables 3 and 4. The cross-resistance factors were calculated from the ratio of the ID_{50} determined for a certain chemotherapeutic drug in GLC-Adr and GLC4-CDDP, respectively, versus the ID_{50} in GLC4. The cross-resistance for DOX in GLC4-Adr was remarkably reduced for both morpholino compounds. This was also the case for camptothecin. GLC4-Adr showed some cross-resistance for methotrexate and cisplatin but was highly cross-resistant for VP-16. GLC4-CDDP was relatively insensitive to the morpholino anthracyclines as well as to camptothecin but was sensitive to DOX, VP-16, and methotrexate.

The effects of pretreatment with BSO on MRA-CN-luced cytotoxicity, expressed as dose-modifying factors at the ID_{50} as determined after continuous incubation in the MTA, were as follows: GLC4, 0.90 ± 0.06; GLC4-Adr, 1.04 ± 0.02; and GLC4-CDDP, 1.18 ± 0.26. Thus, BSO slightly increased MRA-CN-induced cytotoxicity in GLC4-CDDP cells.

Discussion

In our panel of cell lines, MRA-MT and MRA-CN are both very active chemotherapeutic drugs as compared with DOX. Both drugs are most active in GLC4-Adr, the cell line that is about 100-fold resistant to DOX. In this cell line, MRA-CN proved to be 5,790- and 2,590-fold more active than DOX after 1 h and continuous exposure, respectively. This is an interesting observation because of the remarkable properties of GLC4-Adr. Hence, in the MDR H69AR cell line, which also overexpresses MRP [29], Cole [30] reported a relative lack of potency for MRA-CN. However, in this cell line, no DOX-accumulation deficit exists [29]. From our observations the conclusion might be drawn that MRP, just as Pgp-mediated MDR, does not seem to be involved in the sensitivity to MRA-CN. Moreover, these morpholino anthracyclines circumvent altered topoisomerase II activity, as was demonstrated for their cytotoxic activity in the GLC4-Adr cell line. The relative lack of activity found for MRA-MT and, to a lesser degree, also for MRA-CN in GLC4-CDDP cells has not previously been described in a cisplatin-resistant cell line. Ripamonti et al. [13] reported an equivalent efficacy for MRA-MT in the wild-type and in the cisplatin-resistant murine leukemia cell line L1210. A 4-fold MRA-CN-resistant ES-2R cell line, however, also shows 7-fold cross-resistance to cisplatin [31]. The role of an enhanced amount of detoxifying activity, as has been reported for ES-2R as well as for GLC4-CDDP, might play a role in this cross-resistance between MRA-CN and cisplatin [31–33]. This was also partly suggested by the results of our experiments with BSO modulation on MRA-CN-induced cytotoxicity in GLC4-CDDP cells.

Concerning an elucidation of the working mechanisms of MRA-MT and MRA-CN in our cell lines, it is interesting that the cytotoxicity profiles of these drugs in the GLC4 cell lines mimic those of camptothecin and differ markedly from those of VP-16. This confirms earlier observations in which the cytostatic action of morpholino and cyano-morpholino doxorubicin was attributed to DNA topoisomerase I-induced cleavage and not to topoisomerase II-induced cleavage [15]. This finding has to be confirmed in other cell lines.

In our cell lines the activity of MRA-CN was about 2.5-fold that of MRA-MT. Previously, MRA-MT was reported to be 3- to 15-fold more potent than DOX in various cell lines, whereas MRA-CN was 100- to 1,000-fold more potent than DOX [9, 10]. We found an increase in the cytotoxicity of MRA-MT versus DOX that varied between 6- and 1,500-fold. The reason why our observations differ from the previous reports is not clear, as a modest difference in potency between MRA-MT and MRA-CN was observed in all our tested cell lines. The duration of incubation of MRA-CN made no uniform difference in its cytotoxic activity. Althoug the cross-link formation induced by MRA-CN takes place much faster than that caused by, e.g., cisplatin, this apparently has no effect on its final cytotoxic potential. Because of the generally promising cytostatic potency of the morpholino anthracyclines, the results of clinical studies, of which only a few have been reported to date, are awaited with great interest [34, 35].

In conclusion, MRA-MT and MRA-CN are highly potent chemotherapeutic drugs in a DOX-resistant cell line with overexpression of MRP and lowered topoisomerase II activity. Cross-resistance for the morpholino anthracyclines was found in a cisplatin-resistant cell line, suggesting a role for detoxifying systems such as GSH and GST. Topoisomerase I-mediated cytotoxicity is suggested because of the comparable cytotoxicity of the morpholino anthracyclines and camptothecin in the small-cell lung-cancer cell lines. MRA-CN is ca. 2.5-fold more active

than MRA-MT, whereas the duration of incubation does not play a uniform role in its cytotoxic potency.

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Structurally Modified Anthracyclines Retain Activity in a Cell Line with Simultaneous Typical and Atypical Multidrug Resistance

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Abstract. *Resistance to the classical anthracyclines may be due to one or several mechanisms, most notably p-glycoprotein (pGP) associated multidrug resistance (mdr1, "typical mdr") and altered activity of topoisomerase II (topo II) ("atypical mdr"). Modulators of mdr1 will be of limited value in case of combined forms of resistance. A Friend murine erythroleukemia cell line (F4-6R) carrying both mdr1 and topo II mediated anthracycline resistance was used to determine the efficacy of structurally altered anthracyclines against such extended multidrug resistance. Proliferation assays showed that 3'N-morpholinyl substituted anthracyclines were able to retain much of their activity even in this setting. MX2 (KRN8602; 3'-deami-no-3'-[4-morpholinyl]-13-deoxo-10-hydroxycarminomycin), which is 9-alkylated in addition to carrying a 3'N-morpholinyl group, was the most promising agent tested.*

The anthracyclines doxorubicin (DOX) and daunorubicin (DNR) are among the most effective drugs in the battle against cancer. Not rarely, however, their therapeutic effect is diminished or even abolished by the emergence of drug resistance. Classical multidrug resistance associated with the MW 170.000 p-glycoprotein pGP (mdr1), which affects anthracyclines as well as a variety of other "natural" anti-tumor agents, is probably the most intensively studied form of anthracycline resistance (1). Mdr1 is characterized by an active outward transport mechanism, resulting in reduced intracellular drug accumulation (1).

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Key Words: Anthracyclines, multidrug resistance, topoisomerase II, *in vitro*.

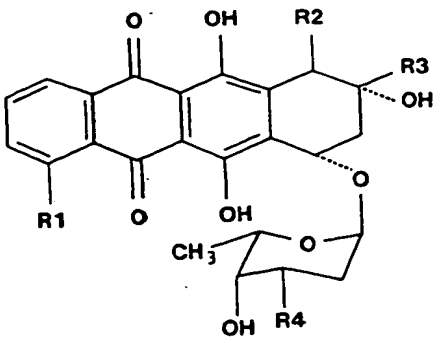
Several approaches have been used to overcome anthracycline resistance. Many preclinical and clinical trials, for instance, have evaluated mdr1 reverting agents such as verapamil or cyclosporin A, which reduce the outward transport of cytostatic agents (2). These mdr1-modulators, however, have a rather narrow spectrum of activity, as they will only achieve their goal if resistance is really due to mdr1. They will not function against other pump proteins, and will also be ineffective if anthracycline resistance is unrelated to drug transport. Such is the case for "atypical" multidrug resistance, which is caused by an alteration of topoisomerase II (topo II) activity (3).

Structurally modified anthracyclines, on the other hand, may not only be able to escape the mdr1 associated pump mechanism, but may also retain their activity in the presence of other forms of resistance (4). Promising agents currently under evaluation include 9-alkylated analogs and substances modified at the amino group of the daunosamine sugar (5). In order to define the activity of such agents in the simultaneous presence of the two probably most important forms of anthracycline resistance (6), we have evaluated a panel of eight anthracyclines in a cell line carrying both "typical", pGP related, and "atypical", topo II related, multidrug resistance.

Materials and Methods

Drugs and chemicals. The anthracyclines DOX, DNR and idarubicin (IDA, 4-demethoxydaunorubicin) were obtained commercially from Farmitalia Carlo Erba, Freiburg, Germany. Aclacinomycin A (ACLA) was purchased from Behringwerke, Marburg, Germany.

Morpholinyldoxorubicin (MRDX, 3'-deamino-3'-[4-morpholinyl]doxorubicin) and cyanomorpholinyldoxorubicin (MRDX-CN; 3'-deamino3'-[3-cyano-4-morpholinyl]-doxorubicin) were generous gifts from Dr. E. Acton (Drug synthesis and chemistry branch, National Cancer Institute, Bethesda, USA). Methoxymorpholinyldoxorubicin (MMRDX) (FCE 23762; 3'deamino-3'-[2(s)methoxyl-4-morpholinyl]-doxorubicin) was kindly provided by Dr. A. Suarato, Farmitalia Carlo Erba, Milan, Italy. MX2 (KRN8602; 3'-deamino-3'-[4-morpholinyl]-13deoxo-10-hy-



	R1	R2	R3	R4
DOX	CH3O	H	COCH2OH	NH2
DNR	CH3O	H	COCH3	NH2
IDA	H	H	COCH3	NH2
ACLA	OH	COOCH3	CH2CH3	
MRDX	CH3O	H	COCH2OH	
MRDX-CN	CH3O	H	COCH2OH	
MMRDX	CH3O	H	COCH2OH	
MX2	OH	OH	CH2CH3	

Figure 1. *Anthracyclines evaluated: Chemical structure. The chemical structures of the eight anthracyclines which were evaluated against F4-6 and F4-6R cells is shown. * In ACLA, the monosaccharide is replaced by a trisaccharide consisting of L-rhodosamine, 2-desoxy-L-fucose, and L-cinerulose. See text for abbreviations.*

droxycarminomycin) was a generous gift from Dr. Y. Toda, Kirin Brewery, Tokyo, Japan. The chemical structure of DOX and the modifications present in the tested anthracycline analogs are presented in Figure 1.

Eagle's minimal essential medium without nucleosides (MEM), L-glutamine and penicillin-streptomycin were obtained from Gibco (Karlsruhe, Germany), fetal calf serum (FCS) from Boehringer (Mannheim, Germany), and trypan blue solution was from Serva (Heidelberg, Germany).

Cell lines. The drug resistant Friend murine erythroleukemia cell line F4-6R, selected by constant exposure to doxorubicin, and its parent line F4-6 were used for all experiments. Both lines were gifts from Dr. Steinheider, Department of Toxicology, University of Hamburg, Germany. They have previously been characterized: in contrast to the parent line F4-6, two mechanisms of multidrug resistance are active in the resistant line F4-6R (7, 8):

1. pGP associated "typical" multidrug resistance (mdr1), as documented by (7, 8):
- A resistance pattern including the classical anthracyclines (DOX and DNR), the epipodophyllotoxins, and vinca alkaloids;
- A reduced net uptake of classical anthracyclines;
- The immunohistochemical detection of pGP by the polyclonal rabbit IgG antibody MDR1 (Oncogene Science Corp., Manhasset, NY, USA);
- Hyperexpression of mdr1 mRNA as shown by Northern blotting using the method described by Chen *et al* (9).
- In addition, mdr1-RNA expression in F4-6R cells could be documented by reverse PCR according to the method described by Noonan *et al* (10) (data not shown).

2. "Atypical" multidrug resistance due to a reduction of topo II activity, as documented by (8):
- The inclusion of the topo II directed drug m-AMSA, which is not subject to transport by pGP, into the resistance spectrum;
- Reduced relaxation of supercoiled plasmid pBR322 DNA by nuclear extract of F4-6R (8).

Table I. *Antiproliferative effects of eight anthracyclines.*

	F4-6 Sensitive cells		F4-6R Resistant cells		
Drug	IC_{50} ng/ml	Relative potency	IC_{50} ng/ml	Relative potency	Resistance factor
DOX	8.7	1.00	1540	1.00	177
DNR	8.0	1.09	680	2.26	85
IDA	3.6	2.42	31	49.68	8.6
ACLA	5.9	1.47	29	53.10	4.9
MRDX	9.3	0.94	39	39.49	4.2
MRDX-CN	0.015	580.00	0.052	29615.38	3.5
MMRDX	1.9	4.58	5.5	280.00	2.9
MX2	7.8	1.12	12.1	127.27	1.6

Synopsis of IC_{50} values and potencies in relation to DOX of modified anthracyclines in sensitive F4-6 and multidrug resistant F4-6R cells, and resistance factors. For definition of "relative potency" and "resistance factor" and for abbreviations see text.

Culture conditions. F4-6 cells were maintained in suspension in MEM supplemented with 10% FCS, 1% L-glutamine and 1% penicillin-streptomycin at 37°C in a humidified atmosphere containing 5% CO_2. Resistant F4-6R cells were propagated under the same conditions, with the only exception of DOX at 1 μg/ml being added to the medium. All cells were grown under drug-free conditions for at least one week before proliferation experiments were performed. Cells used for proliferation assays were collected in the late logarithmic phase of cell growth.

Proliferation assays. All proliferation assays were performed in duplicate with three wells per concentration. For each experiment, 10^6 cells were plated in Petri dishes containing 15 ml medium as described above. Anthracyclines were added at defined concentrations. After seeding, the cells were again incubated at 37°C for 48 hours, then the number of viable cells was counted using the trypan blue exclusion method.

The IC_{50}, defined as the drug concentration causing a 50% decrease of cell proliferation compared to untreated cells, was determined for each drug in each cell line. The relative potency of an anthracycline was calculated by comparing its IC_{50} value to that of DOX. The resistance factor for any of the drugs (RF) was defined as the ratio:

$$\frac{IC_{50} \text{ resistant cell line F4-6R}}{IC_{50} \text{ sensitive cell line F4-6}}$$

Results

A summary of all IC50 values, relative potencies and resistance factors is presented in Table I.

Antiproliferative activity against sensitive F4-6 cells. Proliferation of anthracycline-sensitive F4-6 murine erythroleukemia

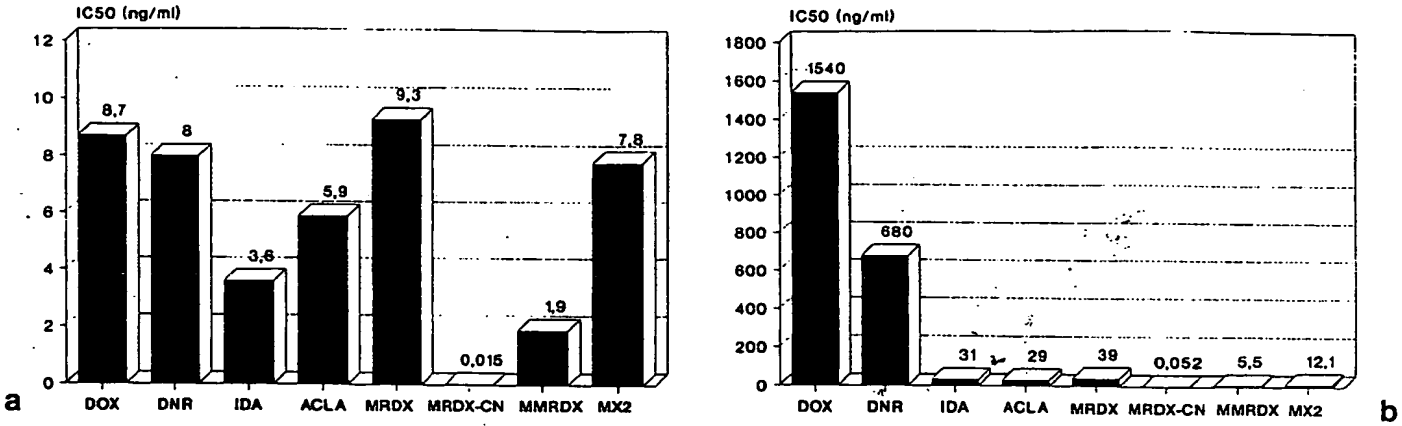


Figure 2. *Inhibition of cell proliferation by anthracyclines a: Sensitive F4-6 cells. b: Resistant F4-6R cells IC_{50} values (ng/ml) for eight anthracyclines as measured in sensitive (a) and"resistant (b) ceils are shown. For abbreviations see text.*

cells was effectively inhibited by all anthracyclines. DOX, added to the medium at a concentration of 8.7 ng/ml, led to 50% inhibition of proliferation (IC_{50}) compared to untreated cells. IC_{50} values in the same order of magnitude were observed for DNR, ACLA, MRDX and MX2, while IDA and MMRDX were 2.4 and 4.6 times more potent than DOX in this drug sensitive line. MRDX-CN was extremely toxic at very low concentrations (580 times as potent as DOX, IC_{50} 0.015 ng/ml). A summary of the effects of various anthracyclines against F4-6 is given in Figure 2a.

Antiproliferative activity against resistant F4-6R cells. In the drug resistant line F4-6R, the IC_{50} of the selecting agent DOX was increased to 1540 ng/ml. DNR was about twice as potent, IDA, ACLA and MRDX were nearly 50 times more active than DOX. Both MX2 and MMRDX were active at concentrations which were over one hundred times lower than DOX. As seen for F4-6, MRDX-CN was the most toxic anthracycline in F4-6R, with an IC_{50} that was almost 30.000 times higher than that of DOX. A summary of the effects of various anthracyclines against F4-6R is presented in Figure 2b.

Comparison F4-6 versus F4-6R. Compared to its sensitive parent line F4-6, the multidrug resistant line F4-6R showed a 177-fold resistance to the selecting agent, DOX. The second classical anthracyline, DNR, had a resistance factor RF of 85. Much lower resistance values were calculated for the other modified anthracyclines: IDA 8.6, ACLA 4.9, MRDX 4.2, MRDX-CN 3.5, MMRDX 2.9. Resistance was almost completely abolished for MX2 (RF=1.6). Resistance factors are shown in Figure 3.

Discussion

Selecting modified anthracyclines. Anthracycline analogs carrying structural modifications are an interesting option when anthracycline resistance is the problem. Compounds carrying 9-alkyl substituents or certain changes involving the amino sugar, namely inclusion of the 3'nitrogen into a morpholinyl ring, have been previously identified as having favorable activity in mdr1 (5, 11, 12). Such substances may also overcome resistance due to non-mdr1 forms of outward drug transport (4) or topo II mediated resistance (13). As an additional benefit, cardiotoxicity may be lower for some analogs (14, 15, 16). However, this has not been proven in carefully designed, controlled clinical trials with adequate follow up.

In the clinical situation, resistance is most likely not confined to a single mechanism, but more than one may be operative at once. Ideally, anthracycline analogs should therefore remain active even if several mechanisms of resistance are present simultaneously. Here, we report our experience with a panel of structurally modified anthracyclines against such extended multidrug resistance due to simultaneous "typical" and "atypical" multidrug resistance within one cell line (combined mdr1/topo II resistance). Compared to drug sensitive F4-6 Friend murine erythroleukemia cells, our resistant line F4-6R was 177-fold resistant to DOX, by which multidrug resistance had been induced. F4-6R was 85-fold resistant to the other classical anthracycline, DNR.

The modified anthracyclines studied included the commercially available 4-demethoxy-analog of DNR, IDA and ACLA, a 9-alkylated anthracycline in which the daunosamine sugar has been replaced by an oligosaccharide. We also evaluated the 3'N-morpholinyl substituted agents MRDX, MRDX-CN, MMRDX and MX2. As stated above, substances of this class seem to be especially well suited to escape resistance (5, 12). MRDX is the 3' N-morpholinyl analog of DOX. MRDX-CN carries an additional cyano group on the morpholinyl ring. This ring is further substituted with a

methoxy group in MMRDX, a substance currently being evaluated in a phase I trial in Scotland (17). MX2, already undergoing phase I/II trials in Japan (18), was of particular interest to us, since it contains, within one molecule, both the structural modifications that Coley *et al* have proposed to be important when dealing with anthracycline resistance (12): the 3' N-morpholinyl ring and the 9-alkylated aglycone.

Activity against sensitive cells. As expected, the various anthracyclines were not uniformly potent even against sensitive F4-6 cells. As shown previously (19), MRDX-CN was the most powerful substance. In F4-6, it was 580 times more toxic than DOX. MMRDX was only about 4½ times more active than DOX against sensitive cells in our *in vitro* assay. Compared to some reported *in vivo* data (potency up to >80 times greater than DOX) (20), the relative potency seems to be quite low. The different observations may be explained by in vivo bioactivation of MMRDX (21), like MRDX (22), to more active metabolites. Accordingly, other researchers have also found MMRDX to be equipotent or only moderately more active than DOX *in vitro* (14, 21).

Conquering resistance. Our main question, however, was: To what extent can anthracycline analogs evade combined mdr1/topo II resistance? As expected, the selecting agent DOX and the second classical anthracycline, DNR, were most heavily affected by resistance. IDA, the more lipophilic 4-demethoxy-analog of DNR, has been advocated as an alternative to DOX and DNR. Its increased lipophilicity has enabled the development of an oral formulation (15). While IDA was able to evade mdr1/topo II resistance to a certain degree in our model, it was still 8.6 times less effective in resistant than in sensitive cells. The 9-alkylated ACLA was somewhat better suited to overcome multifactorial resistance in our model, but considerable resistance (RF 4.9) remained.

Within our panel of eight anthracyclines, the four 3'-N-morpholinyl-analogs were least affected by combined mdr1/topo II resistance. As in p388 leukemia carrying mdr1 (11), MRDX and MRDX-CN retained most of their activity in our mdr1/topo II model. Still, resistance was not completely abolished by MRDX or MRDX-CN in either study. As reported for pure mdr1 (23), MMRDX was more active against combined mdr1/topo II resistance than either MRDX or MRDX-CN (23).

MX2, however, which is not only 3'N-morpholinyl substituted but also 9-alkylated, was the most effective anthracycline in our model of extended multidrug resistance. Very similar effects were seen in sensitive (IC_{50}=7.8 ng/ml) and resistant (IC_{50}=12.1 ng/ml) cells (RF only 1.6). These results confirm data showing remarkably low cross resistance between MX2 and DOX in multidrug resistant lung cancer (24), p388 murine leukemia (25) and several human leukemia cell lines (26, 27, 28). Our study extends these observations to multiple pathway mdr1/topo II resistance.

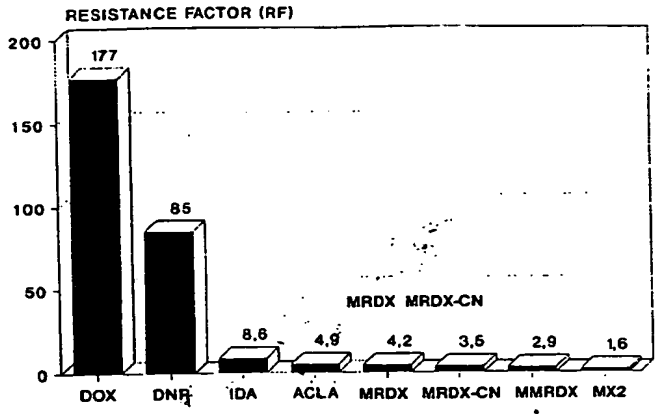


Figure 3. *Resistance factors. Resistance factors of eight anthracyclines. Comparison of IC_{50} values in resistant F4-6R cells to sensitive F4-6 cells.*

How is anthracycline resistance overcome? Why did some anthracyclines remain active in these circumstances where others fail? First, their pharmacokinetic profile may have enabled them to reach and maintain effective intracellular concentrations despite the presence of the active pump mdr1. For example, equivalent intratumor accumulation of MMRDX into sensitive and resistant tumors has recently been demonstrated *in vivo* in a murine model (29). The accomplishment of active intracellular drug levels could be due to a failure of pGP to identify some anthracyclines as well as others as substrates. In support of this hypothesis, 9-alkyl and morpholinyl substituted anthracyclines behaved less favourably as substrates for energy-driven drug efflux by pGP than DOX or DNR in two mdr1 cell lines (30). Also, a faster and greater drug influx balancing outward transport may play a role. Such has been shown for MX2 in mdr1 positive K562/ADM human leukemia cells (28, 31).

Second, it has been well documented that not all anthracyclines inhibit malignant growth by the same mechanism. Different target sites and different modes of action may be relevant. For example, topo II seems to be an important target for DOX (32) and maybe even more so for IDA (33). Neither MRDX nor MRDX-CN, however, as representative examples of 3'N-morpholinyl anthracyclines, stimulated DNA-cleavage induced by purified mouse leukemia topo II (34). Accordingly, MRDX and MMRDX retained activity in the human CEM/VM-1 leukemia cell carrying topo II mediated atypical multidrug resistance (13). On the other hand, in contrast to the classical anthracyclines, MRDX-CN is a powerful DNA-DNA intercalator (35, 36). Therefore, some (morpholinyl) anthracyclines might evade topo II mediated resistance simply because topo II is not one of their targets.

Applying these thoughts to our results, we can assume that the morpholinyl anthracyclines, and of these especially MX2, evaded mdr1 to reach adaequate drug levels intracellularly,

where they then acted preferentially on targets other than topo II.

Conclusions

Modified anthracyclines which are 3'N-morpholinyl substituted retain much of their activity even in the presence of extended multidrug resistance due to the simultaneous expression of both mdr1 and altered topo II activity. MX2, which not only carries a 3'N-morpholinyl group, but is also alkylated in position 9 of the aglycone, was the most promising substance in this *in vitro* setting.

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ORIGINAL ARTICLE

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Differential single- versus double-strand DNA breakage produced by doxorubicin and its morpholinyl analogues

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Abstract The morpholinyl analogues of doxorubicin (DOX) have previously been reported to be non-cross-resistant in multidrug resistant (MDR) cells due to a lower affinity for P-glycoprotein relative to the parent compound. In order to further investigate the mechanisms of action of these morpholinyl anthracyclines, we examined their ability to cause DNA single- and double-strand breaks (SSB, DSB) and their interactions with topoisomerases. Alkaline elution curves were determined after 2-h drug treatment at 0.5, 2 and 5 μM, while neutral elution was conducted at 5, 10 and 25 μM in a human ovarian cell line, ES-2. A pulse-field gel electrophoresis assay was used to confirm the neutral elution data under the same conditions. Further, K-SDS precipitation and topoisomerase drug inhibition assays were used to determine the effects of DOX and the morpholinyl analogues on topoisomerase (Topo) I and II. Under deproteinated elution conditions (pH 12.1), DOX, morpholinyl DOX (MRA), methoxymorpholinyl DOX (MMDX) and morpholinyl oxaunomycin (MX2) were equipotent at causing SSB in the human ovarian carcinoma cell line, ES-2. However, neutral elution (pH 9.6) under deproteinated conditions revealed marked differences in the degree of DNA DSB. After 2-h drug exposures at 10 μM, DSBs were 3300 rad equivalents for MX2, 1500 for DOX and 400 for both MRA and MMDX in the ES-2 cell line. Pulse-field data substantiated these differences in DSBs, with breaks easily detected after MX2 and DOX treatment, but not with MRA and MMDX. DOX and MX2 thus cause DNA strand breaks selectively through interaction with Topo II, but not Topo I. In contrast, MRA and MMDX cause DNA breaks through interactions with both topoisomerases with a predominant inhibition of Topo I.

Key words Anthracyclines · DNA strand breakage · Morpholinyl doxorubicin · MX2 · topoisomerases

Introduction

The antineoplastic agent, doxorubicin (DOX), is important clinically, but its use is limited due to cardiotoxicity, myelosuppression and multidrug resistance (MDR). In an attempt to overcome this drug resistance, Acton et al. [1] synthesized a series of DOX derivatives with morpholinyl groups at the 3′ position of the sugar moiety. These DOX analogues are non-cross-resistant in MDR variants in vitro, and have been shown to increase cellular accumulation of drug relative to the parent compound by tritiated and fluorescent assays [2–5]. These data suggest that the addition of the morpholinyl group alters the affinity of DOX for P-glycoprotein, an ATP-dependent efflux pump encoded by the *mdr*1 gene [6–10]. Also, these DOX analogues have been shown to be more lipophilic than DOX [1] and non-cardiotoxic at antitumor doses [2, 3]. Examples of these morpholinyl derivatives include morpholinyl DOX (MRA), methoxymorpholinyl DOX (MMDX), and morpholinyl oxaunomycin (MX2) (Fig. 1).

Previously, we have demonstrated that the MRA and MMDX compounds can be activated by microsomal metabolism and can crosslink DNA [11, 12]. This activation is associated with a potentiation of their cytotoxicity in vivo and in vitro [13, 14]. The closely related but extremely potent cyanomorpholinyl

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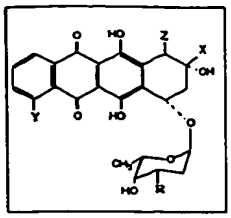

Name	Abbreviation	X	Y	Z	R
1. Doxorubicin	DOX	$-C(=O)-CH_2OH$	$-OCH_3$	-H	$-NH_2$
2. Morpholinyl doxorubicin	MRA	$-C(=O)-CH_2OH$	$-OCH_3$	-H	morpholinyl
3. Morpholinyl oxaunomycin	MX2	$-CH_2CH_3$	-OH	-OH	morpholinyl
4. Methoxymorpholinyl doxorubicin	MMDX	$-C(=O)-CH_2OH$	$-OCH_3$	-H	methoxymorpholinyl

Fig. 1 The chemical structures of DOX and the morpholinyl anthracyclines

derivative (MRA-CN) is capable of crosslinking DNA without microsomal activation [2]. We report here a further study of these native morpholinyl DOX compounds (i.e. no microsomal bioactivation). DNA elution assays were used in order to quantify the degree of single-strand breakage (SSB) and double-strand DNA breakage (DSB) induced after a 2-h treatment in a drug-sensitive human ovarian cell line relative to the parent compound, and the results were confirmed with a pulse-field gel electrophoresis assay. Further, data from K-SDS precipitation assays for protein–DNA complexes and topoisomerase activity inhibition experiments suggest that MRA and MMDX induce DNA strand breaks through interaction with topoisomerase I (Topo I), while the mechanism of action for MX2 appears to be more similar to DOX and related to the inhibition of topoisomerase II (Topo II).

Materials and methods

Drugs

MRA was generously provided by Dr. E. M. Acton (Drug Synthesis and Chemistry Branch, National Cancer Institute). MMDX was obtained from Farmitalia Carlo Erba Laboratories (Milano, Italy). MX2 was provided by the Kirin Company (Japan) and DOX was purchased as a commercial preparation from Adria Laboratories (Columbus, Ohio). Drug stock solutions were prepared in absolute ethanol at a concentration of 1.0 m*M* and stored at −20 °C.

Cell culture

The human ovarian carcinoma cell line, ES-2, was established in our laboratory and was grown as a monolayer culture in McCoy's 5A medium supplemented with 10% newborn calf serum, 0.3 mg/l glutamine, 100 U/ml penicillin/ml, and 100 mg/l streptomycin (all from GIBCO Laboratories, Grand Island, N.Y.). ES-2 cells were free of mycoplasma contamination as determined by the GEN-Probe hybridization assay (GEN-Probe, San Diego, Calif).

MTT cytotoxicity assay

The cytotoxicity of DOX and the morpholino anthracyclines was determined using a modified MTT 3-(4,5-dimethylthiazol(-2-yl,1-2,5-diphenyl-tetrazolium bromide assay [15]. ES-2 cells were plated in 96-well microtiter plates (Falcon, Becton Dickinson Co.; Lincoln Park, N.J.) in 200 µl medium. After 24 h, the cells were exposed to drugs at the appropriate dilutions and allowed to incubate for an additional 48 h (approximately two cell divisions). (MTT 20 µl, of MTT 5 mg/ml in phosphate-buffered saline) was added to each well and incubated at 37 °C in a humidified atmosphere containing 5% CO_2. After 3 h, the medium was aspirated and 0.1 *N* HCl-isopropanol solution was added in order to solubilize the formazan crystals. Absorbances were read at 570 nm on a Molecular Devices U.V. Thermomax multiwell spectrophotometer (Molecular Devices, Menlo Park, Calif). Each drug was tested in quadruplicate and in at least three different experiments. Initial experiments indicated that 48 h was suitable for measuring cytotoxicity, as further drug incubation did not result in enhanced cytotoxicity.

Alkaline elution

The alkaline elution technique employed was modified from the method of Kohn [16]. Briefly, ES-2 cells were labeled for 36–48 h with 0.01 µCi/ml of [methyl-^{14}C]-thymidine and internal standard cells were labeled with 0.1 µCi/ml [methyl-^{3}H]-thymidine (both from Amersham Corporation, Arlington Heights, Il). The cells were chased with cold medium overnight and then exposed to drug for 2 h at 37 °C. In order to calculate radiation equivalents, one flask of ^{14}C-labeled cells was irradiated with 300 cGy using a mark 1, model 25 cesium-137 gamma-irradiating machine (J. L. Shepherd & Associated, Glendale, Calif). Internal control cells labeled with ^{3}H were irradiated with 400 cGy. Approximately 10 000 cpm ^{14}C-labeled cells and 20 000 cpm ^{3}H-labeled cells were loaded onto a smoke stack column with a 0.8 µm vinyl/acrylic copolymer filter (DM Metricel filter, 25 mm; Gelman Sciences, Ann Arbor, Mich). Cells were lysed by adding 2% SDS, 0.02 *M* EDTA at pH 10.0 in the presence and absence of 0.5 mg/ml proteinase K (Sigma Chemical Co., St. Louis, M.) for 30 min; the lysate on the filter was washed with 0.02 *M* EDTA, pH 10.0. The elution buffer consisted of 0.02 *M* EDTA and 0.1% SDS, adjusted to pH 12.1 with tetrapropylammonium hydroxide (Eastman Kodak Co., Rochester, N.Y.).

A Manostat cassette pump (New York, N.Y.) was employed to provide an elution rate of 2 ml/h and 4-ml elution fractions were collected using an LKB SuperRac fraction collector (LKB-Produkter, Bromma, Sweden). Ecolite scintillation cocktail (ICN Biomedicals, Irvine, Calif.) was added to each fraction, as well as to the 1 *N* HCl-treated filter. Dual radioactivity was counted using an LS-8000 counter (Beckman Instruments, Palo Alto, Calif.). Log-fractions of ^{14}C retained against ^{3}H retained were plotted using an Excel program on a Macintosh computer.

Neutral elution

The alkaline elution procedure was followed with the following modifications. ^{14}C-labeled ES-2 cells were irradiated with 3000 cGy and ^{3}H-labeled cells with 5000 cGy. The elution buffer consisted of 0.02 *M* EDTA and 0.1% SDS, adjusted to pH 9.6 with tetrapropylammonium hydroxide for the detection of DSBS.

Asymmetric field inversion gel electrophoresis (AFIGE)

A modified AFIGE technique was followed for the quantification of DNA DSBs induced in ES-2 cells [17]. ES-2 cells were labeled with [methyl-^{14}C]-thymidine for 24 h followed by 2-h drug incubations. Cells were then cast in a 1% insert agarose (Seakem), and lysed in a 1% sodium lauryl sarkosine, 0.5 *M* EDTA and 1 mg/ml proteinase K solution at 50 °C overnight. The cells were treated with 100 μg/ml DNase-free ribonuclease A for 4 h at 37 °C. The 0.8% (w/v) agarose gel in 0.5 × Tris-borate/boric acid (TBE) buffer was run at 900s forward pulse time at 1.25 V/cm and 75 s backward pulse time at − 2.5 V/cm for a total electrophoresis time of 33 h. The agarose plugs were then removed from the gel, treated with 10 *N* HCl and melted on a hotplate. Ecolite scintillation cocktail was added to each and read in a Beckman LS-8000 counter. Quantification of DNA DSBs was determined by calculating the percentage of DNA released from the agarose plugs using an Excel program on a Macintosh computer.

K-SDS precipitation assay

The topoisomerase assay of Rowe et al. [18] was used with some modification. Briefly, ES-2 cells were labeled with 1 μCi/ml of [methyl-^{3}H]-thymidine for 24 h. Cells were then washed once in PBS and exposed to the appropriate drug dilution (10 μ*M* for each cytotoxin tested) for 1 h at 37 °C in an atmosphere containing 5% CO_2. The medium was subsequently aspirated and cells lysed with a 1.25% SDS, 5 m*M* EDTA (pH 8.0) supplemented with 0.4 mg/ml salmon sperm DNA. After shearing the DNA, cell lysates were transferred to 1.5-ml Eppendorf tubes containing 200 μl 325 m*M* KCl. Each tube was maintained at 65 °C for 5 min and then cooled on ice for an additional 10 min. Tubes were then spun at 10000 *g* for 2 min at room temperature in an Eppendorf centrifuge. The supernatants were aspirated and pellets washed twice with 10 m*M* Tris-HCl (pH 8.0), 100 m*M* KCl, 1 m*M* EDTA, supplemented with 0.1 mg/ml salmon sperm DNA. The suspension was cooled on ice and spun at 10000 *g*. The pellet was resuspended in 500 μl H_2O and maintained at 65 °C until the pellet was dissolved. After the suspension was transferred to a scintillation vial, 10 ml Ecolite cocktail was added to each and was counted in a Beckman LS-8000 counter. The percentage of specifically precipitated DNA was calculated using an Excel program on a Macintosh computer as follows:

$$\% \text{ precipitated} = \frac{\text{Counts}_{\text{tested}} - \text{Counts}_{\text{control}}}{\text{Counts}_{\text{plated}} - \text{Counts}_{\text{control}}} \times 100$$

Topoisomerase I/II drug screening

The induction of cleavage complexes by Topo I and II was studied using drug screening assay kits purchased from TopoGEN (Columbus, Ohio). Briefly, purified human DNA Topo I (10 units) was incubated with 0.25 μg of supercoiled *pHOT* plasmid (form I DNA) with a specific cleavage site for Topo I in a buffer containing 10 m*M* Tris-HCl (pH 7.5), 1 m*M* EDTA and 100 m*M* NaCl. Drugs were added to the mixture to final concentrations of 0.1, 0.5, 0.75 and 1 μ*M* and incubated at 37 °C for 30 min in a Perkin Elmer 9600 DNA thermal cycler (Norwalk, Ct.). Camptothecin (CPT) was used as a positive control drug for the inhibition of Topo I activity. The reaction mixture (20 μl) was stopped by the addition of 10% SDS and proteinase K (50 μ g/ml) followed by extraction with chloroform and isoamyl alcohol (24: 1 v/v).

For the Topo II assays, 4 units of human Topo II was incubated with a supercoiled DNA substrate (*pRYG* DNA) containing a single Topo II cleavage site in the presence and absence of drug for 30 min at 37 °C. VM-26, a known Topo II inhibitor, was used as a positive control. The buffer for the Topo II assays consisted of 30 m*M* Tris-HCl (pH 7.6), 3 m*M* ATP, 15 m*M* mercaptoethanol, 8 m*M* $MgCl_2$ and 60 m*M* NaCl. The assay was extracted as described above and the samples were then analyzed by agarose (1%) gel electrophoresis at 1.5 V/cm in 4 × Tris-acetate/EDTA (TAE) buffer. DNA was visualized by ethidium bromide staining.

Results

The in vitro cytotoxicity data for DOX and its morpholinyl derivatives in the ES-2 cell line are summarized in Table 1. MMDX was the most potent, followed by MRA and DOX, while MX2 was only half as potent as the parent compound. The three morpholinyl drugs were non-cross-resistant in our DOX-selected MDR variants [5].

In order to further investigate the mechanism(s) of action for these compounds, DNA elution studies were conducted and the alkaline elution profiles of ES-2 cells are shown in Fig. 2. After 2-h drug incubations, protein-associated DNA SSBS appeared to peak at 2 μ*M* for DOX, MMDX and MX2, while MRA continued to show a linear increase in DNA SSBs at 5 μ*M*. With the exception of MRA, DOX and its morpholinyl derivatives appeared to reach a maximum number of SSBs at approximately 200 rad equivalents (Table 2). The neutral elution profiles under deproteinated conditions are shown in Fig. 3. Interestingly, MX2 induced the greatest number of protein-associated DNA DSBs in the ES-2 cell line, followed by DOX. MRA and MMDX induced only a fraction of the DSBs as compared to the parent compound or MX2. MX2 at 25 μ*M* surpassed the 5000 cGy control in its ability to cleave DNA. DOX, on the other hand, reached its maximum DSBs at 10 μ*M*, producing approximately 1500 rad equivalents (Table 3). MRA and MMDX both reached a plateau at 10 μ*M* with approximately 300 rad equivalents.

To verify the results found with neutral elution, we employed the technique of AFIGE or pulse-field gel electrophoresis. The same drug concentrations were used in ES-2 cells for 2-h drug incubations in order to determine the percentage of DNA released under deproteinated conditions. The pulse-field gel profiles substantiated our elution data (Fig. 4), demonstrating that MX2 was clearly the most potent in causing DNA DSBs. DOX-induced DSBs were significant, while strand breaks in ES-2 cells treated with MRA and MMDX were hardly detectable in this assay. In these experiments, however, all compounds appeared to have induced the maximum number of DSBs at 5 μ*M*, with 18% DNA released from the agarose plugs in cells treated with MX2 (Table 4). DOX resulted in approximately 14% DNA released, while both MRA and MMDX released only 1.8 and 1.6%, respectively. Although this assay substantiated our results in terms of the total amount of DNA released post-drug treatment,

Table 1 Cytotoxicity of DOX and the morpholinyl anthracyclines in the ES-2 cell line after 48 h drug exposure. Each IC_{50} value represents the mean of at least four experiments ± SD

Drug	IC_{50} (n*M*)[1]	Potency ratio[a]
DOX	78 ± 7.0	1.0
MRA	29 ± 5.0	2.7
MMDX	2.7 ± 0.3	29
MX2	170 ± 19	0.5

[a] Relative to the IC_{50} for DOX

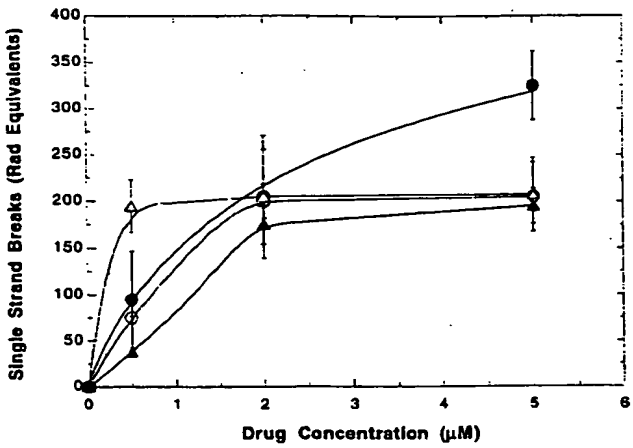

Fig. 2 The alkaline elution (pH 12.1) profiles of ES-2 cells treated with DOX (O), MRA (O), MMDX (Δ) or MX2 (Δ) under deproteinated conditions

Table 2 DNA single strand breaks induced in the ES-2 cell line after 2-h exposures to DOX and the morpholinyl anthracyclines. The data are expressed as rad equivalents. Each value represents the mean of at least two experiments ± SD

	Drug			
Concentration (μ*M*)	DOX	MRA	MMDX	MX2
0.5	78 ± 1.4	92 ± 52	200 ± 28	39 ± 2.1
2.0	200 ± 18	210 ± 51	210 ± 66	170 ± 21
5.0	220 ± 37	340 ± 37	220 ± 40	200 ± 19

the neutral elution technique provided a better method of quantitating the degree of DNA DSB produced by these compounds because of its ability to measure DNA DSB over a time course (five fractions over 10 h). In this way, one could detect subtle differences after various stages of DNA elution impossible using the AFIGE technique.

Therefore, DOX, MX2, MRA and MMDX all cause protein-associated DNA SSBs to the same degree, but MX2 and DOX are more potent at causing DNA DSBs. These results suggest that MRA and MMDX

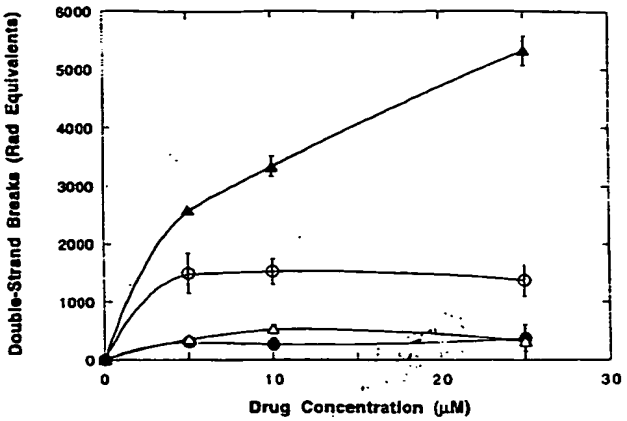

Fig. 3 The neutral elution (pH 9.6) profiles of ES-2 cells treated with DOX (O), MRA (O), MMDX (Δ) or MX2 (Δ) under deproteinated conditions

Table 3 DNA double strand breaks induced in the ES-2 cell line after 2-h exposures to DOX and the morpholinyl anthracyclines. The data are expressed as rad equivalents. Each value represents the mean of at least two experiments ± SD

	Drug			
Concentration (μ*M*)	DOX	MRA	MMDX	MX2
5	1490 ± 350	306 ± 31	355 ± 32	2590 ± 19
10	1520 ± 220	270 ± 110	529 ± 71	3340 ± 180
25	1370 ± 270	370 ± 230	315 ± 76	5320 ± 250

may cause DNA strand breaks through interaction with Topo I, and that both MX2 and DOX interact with Topo II. K-SDS assays for the quantification of covalently linked Topo I- and II-DNA precipitates were consistent with this hypothesis. MRA and MMDX precipitated significant amounts of DNA linked to protein in whole ES-2 cell preparations relative to CPT, a compound known to interact with Topo I (Fig. 5). Likewise, MX2 and DOX precipitated significant amounts of DNA relative to VP16 used as a positive control.

Further, topoisomerase drug screening assays also suggested that MRA and MMDX interact with Topo I. MRA and MMDX stimulated the formation of cleavable complexes in a dose-dependent fashion, resulting in an increase in the amount of nicked, open circular form of DNA. Also, these drugs inhibited the conversion of supercoiled (form I) *pHOT* DNA to relaxed DNA topoisomers normally seen after Topo I incubation (Fig. 6), while incubation with DOX and MX2 had no effect at the same drug concentrations. Treatment with CPT resulted in an increase in the amount of open circular DNA from relaxed topoisomers at 0.1 m*M*.

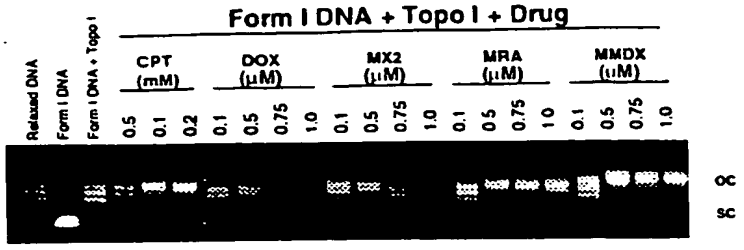

Fig. 6 *pHOT* plasmid (form I DNA) exposed to human topoisomerase I ± DOX or the morpholinyl anthracyclines (0.1, 0.5, 0.75 and 1.0 μ*M*). CPT was used as a positive control for Topo I inhibition (0.05 to 0.2 m*M*)

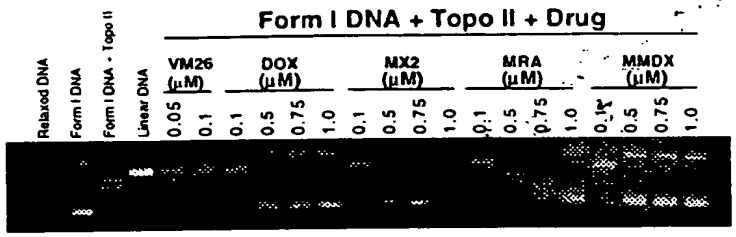

Fig. 7 Supercoiled *pRYG* DNA exposed to human Topo II ± DOX or the morpholinyl anthracyclines (0.1, 0.5, 0.75 and 1.0 μ*M*). VM26 was used as a positive control for Topo II inhibition (0.05 and 0.1 μ*M*)

higher levels of SSB than DOX at lower drug concentrations (Tables 1, 2; Fig. 2). Specifically, MMDX induced 2.5 times higher SSB than DOX at 0.5 μ*M*, which was reflected in MTT assays where MMDX was 29 times more potent than the parent compound. Likewise, MRA induced higher amounts of SSB at the higher concentration of 5.0 μ*M*, and was 2.7 times more potent than DOX after 48 h drug incubation in our cytotoxicity assays.

The MRA and MMDX compounds failed to induce any significant DSB in our neutral elution and pulse-field electrophoresis assays. In contrast, DSBs induced by MX2 and DOX were readily detectable by both techniques. This difference in SSBs and DSBs indicates that MRA and MMDX may work primarily through Topo I since the dominant DNA lesion produced was an SSB. Indeed, our drug inhibition topoisomerase assays provide direct evidence that MRA and MMDX cleavage is associated with Topo I, and that MX2 cleavage is associated with Topo II.

Wasserman et al. have previously reported on the effects of MRA on purified mouse leukemia L1210 DNA Topo I [23]. MRA treatment (1–2 μ*M*) resulted in *fok*I DNA cleavage at position 4955 as well as two novel areas at positions 4975 and 5007 relative to the cleavage pattern usually observed post-CPT exposure. MRA also suppressed cleavage at position 4997 in a manner similar to CPT, with total suppression of Topo I-mediated cleavage at higher concentrations in a dose-dependent manner. In similar assays with purified Topo II, MRA failed to have any effect, while DOX stimulated Topo II cleavage and failed to induce Topo I-associated strand breaks. The cyanomorpholinyl derivative also failed to have any inhibitory effects on this enzyme in the study by Wasserman et al., but stimulated Topo II-mediated cleavage in a manner similar to DOX under the same conditions. The presence of the α-cyano group on the morpholinyl moiety results in a completely different mechanism of action. MRA-CN causes DNA–DNA crosslinks in the ES-2 cell line quite like the bioactivated MRA and MMDX compound upon incubation with human liver microsomes in the presence of NADPH [24]. Further, Capranico et al. found that the morpholinyl group must be at the 3′ position in order to form cleavable complexes since derivatives with the moiety at the 4′ position of DOX failed to stimulate DNA cleavage and trap topoisomerase [25].

There are other fundamental differences between DOX and the morpholino anthracyclines. Data suggest that morpholinyl substitution has a profound effect on ribosomal gene transcription [26]. In contrast to studies with DOX, MRA has been reported to have potent inhibitory effects on ribosomal RNA transcription, while the Topo I inhibitor CPT has been shown to inhibit the synthesis of the 45 S rRNA precursor [27]. These effects have been observed in another antineoplastic agent, actinomycin-D, which induces Topo I-associated DNA strand breaks [23] and has been shown to inhibit ribosomal gene transcription [25]. Thus, there may be a significant correlation between the

effects on Topo I and the inhibitory effects on rRNA transcription.

Finally, since the double-strand lesion is generally considered to be the most lethal, one might expect that MX2 would be the most potent compound in our cytotoxicity testing, since it was the most potent in causing DNA DSBS. Yet, MX2 was only half as potent as DOX in vitro. Although the reasons for this discrepancy are not known, possible explanations include differential repair of MX2 DNA damage and different specificity for DNA sequences or topology. These experiments provide further evidence that minor alterations either on the morpholinyl moiety or on the anthracycline profoundly affect the interaction of these compounds with DNA and topoisomerases.

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Cancer. 1988 Aug 1; 62(3): 479-83.

Doxorubicin versus no antitumor therapy in inoperable hepatocellular carcinoma. A prospective randomized trial.

Lai CL, Wu PC, Chan GC, Lok AS, Lin HJ.

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To assess the efficacy and safety of Adriamycin (Adria Laboratories, Columbus, OH) in inoperable hepatocellular carcinoma (HCC), 60 patients were randomized to receive Adriamycin 60 to 75 mg/m2 at 3-week intervals and 46 patients to receive no antitumor therapy. The median survival rate of the Adriamycin group was 10.6 weeks; that of the group receiving no antitumor therapy was 7.5 weeks (P = 0.036). Adriamycin induced tumor regression of 25% to 50% in 5% of patients and of over 50% in only 3.3% of patients. It caused fatal complications (septicemia and cardiotoxicity) in 25% of patients. The severity of neutropenia leading to septicemia for a particular dose was unpredictable. Four of eight patients who developed cardiotoxicity received less than 500 mg/m2 of Adriamycin. We conclude that Adriamycin is not an ideal drug for the treatment of inoperable HCC.

Publication Types:

- Clinical Trial
- Randomized Controlled Trial

PMID: 2839280 [PubMed - indexed for MEDLINE]

Reg Cancer Treat 3(4): 197-201, 1990

Treatment of primary hepatocellular carcinoma by hepatic arterial infusion of 4'-epirubicin (Eng).

Shepherd FA; Rotstein L; Blackstein ME; Burkes R; Erlichman C; Iscoe N; Kutas G; et al:

A group of 23 patients (20 male, 3 female) with hepatocellular carcinoma were treated by hepatic arterial infusion of 4'-epirubicin every 4 weeks. At each treatment, a catheter was inserted percutaneously into the main hepatic artery via the femoral artery under image intensification. Treatment consisted of a 24-h continuous HAI of epirubicin, 30 mg/msup 2/day for 3 days, without heparin. Eleven patients had only one infusion, 4 patients two infusions, 2 patients three infusions, 2 patients four infusions, and 1 patient six and eight infusions each. A partial response was seen in 3 patients, median duration 16 weeks (range 12-46 weeks). Seven patients remained stable, median duration 13 weeks (range 4-38 weeks). The median survival of the overall group was 18 weeks. Survival of responding, stable, and non-responding patients were 38 weeks, 19 weeks, and 10 weeks, respectively. Complications of catheter placement included asymptomatic dissection of the hepatic artery (3 patients), and asymptomatic thrombosis of the hepatic artery (3 patients). Eight patients experienced moderate nausea and vomiting, and 11 patients had moderate to severe alopecia. The granulocyte nadir was above 1000 mul in 83% of evaluable courses, 500-1000 mul in 6%, and less than 500 mul in 11% of courses. Two patients developed neutropenia-associated fever. A platelet nadir below 100,000/mul was seen after only 8% of courses, and only 1 patient had platelets below 50,000/mul. In conclusion, epirubicin has modest activity in hepatocellular carcinoma and is well tolerated when given by hepatic arterial infusion.

Semin Oncol. 1997 Apr;24(2 Suppl 6):S6-18-S6-25.

Adjuvant chemotherapy with epirubicin and carmofur after radical resection of hepatocellular carcinoma: a prospective randomized study.

Ono T, Nagasue N, Kohno H, Hayashi T, Uchida M, Yukaya H, Yamanoi A.

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The intrahepatic recurrence rate is extremely high, even after radical resection of hepatocellular carcinoma (HCC). One report showed intra-arterial administration of epirubicin to be effective in the treatment of nonresectable HCC. We evaluated the effect of postoperative adjuvant chemotherapy including this drug. Fifty-seven patients who had undergone radical resection of HCC were entered in this study. Using the sealed-envelope method, 27 patients were assigned to a group undergoing resection only and 29 patients to a group also receiving adjuvant chemotherapy after resection. The protocol of the chemotherapy was intra-arterial administration of epirubicin (40 mg/m2) at 1 month after resection followed by intravenous administration of epirubicin (40 mg/m2) every 3 months for 2 years. In addition, 1-hexylcarbamoyl-5-fluorouracil (HCFU; carmofur), 300 mg/d, was administered orally every day, beginning from 1 month after the resection and continuing (in principle) for 24 months. The clinicopathologic backgrounds were well randomized between the two groups. The chemotherapy protocol was performed completely in only five patients (7.2%). Twenty-four (82.8%) patients had to cease the protocol due to various reasons: change to a new therapy after recurrence of HCC in 19 cases (79.2%), severe side effects of the chemotherapy in three cases (12.5%), liver failure in one case (4.2%), and a postoperative complication in one case (4.2%). The mean total doses were 128 +/- 114 mg for epirubicin and 144 +/- 84 g for HCFU. The cumulative overall and disease-free survival rates for 5 years were not significantly different between the two groups. The results of this prospective randomized study suggest that this adjuvant chemotherapy protocol with epirubicin and HCFU after radical resection of HCC was not effective

Publication Types:

- Clinical Trial
- Randomized Controlled Trial

PMID: 9151912 [PubMed - indexed for MEDLINE]

(Meeting abstract) (Eng). Ann Oncol 5(Suppl 8)1994

Arterial chemoembolization with epirubicin in unresectable hepatocellular carcinoma in cirrhosis

Colleoni; Gaion; Liessi; Mastropasqua; Nelli; Sgarbossa; Manente:

No reliable therapies have yet been established for unresectable hepatocellular carcinoma (HCC). Systemic chemotherapy with anthracyclines gives less than 20% objective remissions. Encouraging data in terms of response rate and survival have recently been reported with intra-arterial chemotherapy alone or combined with various veno-occlusive materials, specifically ethiotized oil and gelatin sponge; To evaluate the activity and tolerance of a new chemoembolization protocol, patients with unresectable HCC in cirrhosis were treated with epirubicin (50 mg) and ethiotized oil (10-15 ml), administered through hepatic arterial catheters placed percutaneously during angiography, followed by gelatin sponge. Therapy was repeated for a maximum of 3 cycles. Twenty-two eligible patients have entered the study and are evaluable for response and toxicity. Patients were not pretreated with chemotherapy, and only 1 patient had been submitted to surgery. Patient characteristics were: median ag 70 yr (range 59-77); ECOG performance status 0-1 in 15 and 2 in 7 cases; Child's A disease in 11 and B in 11; Okuda Stage I in 12 and Stage II in 1 cases; TNM Stage II in 9, Stage III in 3 and Stage IVA in 10 cases. Histologically documented cirrhosis was present in all cases (10 alcohol correlated, 4 Hb-sAg correlated, and 8 HCV related).

A total of 53 courses of therapy have been delivered. Three partial remissions (13%), 2 stabilizations of disease and 17 progressions have been observed. Median time to progression was 4 mo with a median survival of 7.6 mo (range, 1-26+ mo). Significant differences in survival (p less than 0.0001) have been observed between patients at Stage II-III (20 mo) and those at stage IVA (3 mo). The treatment was well tolerated with only 2 cases of WHO Grade I pain and 2 cases of Grade I fever. In conclusion, our results indicate that the schedule has only limited activity in HCC and does not seem to offer any sure advantage over other treatments modalities in HCC.

ORIGINAL ARTICLE

Postoperative Adjuvant Chemotherapy After Curative Resection of Hepatocellular Carcinoma

A Randomized Controlled Trial

Edward C. S. Lai, MS; Chung-Mau Lo, MBBS(HK); Sheung-Tat Fan, MS; Chi-Leung Liu, MBBS(HK); John Wong, PhD, DSc

Objective: To study the effect of adjuvant chemotherapy after curative hepatic resection in patients with hepatocellular carcinoma.

Design: A randomized controlled trial.

Setting: A tertiary referral center.

Patients: During a 54-month period, 142 patients with hepatocellular carcinoma underwent hepatic resection at 1 institution. Sixty-six patients who survived the operation and had no demonstrable evidence of residual disease on ultrasonographic examination and hepatic angiographic testing at 1 month after surgery agreed to participate in the study. The median follow-up time was 28.3 months.

Intervention: Thirty patients received a combination of intravenous epirubicin hydrochloride (8 doses of 40 mg/m^2 each at 6-week intervals) and transarterial chemotherapy using an emulsion of iodized oil and cisplatin (3 courses with a maximum dose of 20 mL each at 2-month intervals). Thirty-six patients had no adjuvant treatment.

Main Outcome Measures: Recurrence rate and disease-free survival.

Results: A total of 138 courses of intravenous epirubicin was given to the 30 patients. Sixty-one courses of transarterial chemotherapy were given to only 29 of the 30 patients assigned to the treatment group, because the hepatic artery in 1 patient was thrombosed. Six patients (20%) had chemotherapy-related complications with no mortality. Twenty-three of 30 patients in the treatment group and 17 of 36 patients in the control group had recurrences (P=.01). Patients who received adjuvant chemotherapy had a higher incidence of extrahepatic metastases (11 patients vs 5 patients; P=.03). The respective disease-free survival rates at 1, 2, and 3 years were 50%, 36%, and 18% for the treatment group and 69%, 53%, and 48% for the control group (P=.04).

Conclusion: In a group of patients who underwent curative resection of hepatocellular carcinoma, postoperative adjuvant chemotherapy using the present regimen was associated with more frequent extrahepatic recurrences and a worse outcome.

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ALTHOUGH THE safety of hepatectomy for patients with hepatocellular carcinoma has improved,[1] the prognosis of these patients remains guarded as recurrences are frequent. Depending on the size of the primary tumors, recent reports from Japan,[2-5] France,[6,7] and Hong Kong[1] showed that recurrent disease could be found in 20% to 64% of these patients within the first year and 57% to 81% at 3 years after surgery. While the hepatic remnant was the predominant site of recurrence, involvement of extrahepatic organs such as lung and bone was frequent.[1,8] To improve the long-term outcome of these patients after a successful resection, effective measures to reduce the risk for recurrence are mandatory. Preoperative transarterial chemoembolization has demonstrated no significant benefit and may accelerate deterioration of the already compromised liver function in patients with cirrhosis.[9] Recent retrospective studies showed encouraging results with the use of postoperative adjuvant chemotherapy in the prevention of recurrent disease.[2,10-13] Either the transarterial or systemic route was used and various chemotherapeutic agents, including fluorouracil, mitomycin, cisplatin, and doxorubicin and its derivatives had been used in combination or as a single agent. The regimens were extremely varied and questions such as the exact choice and dosage of anticancer agents, optimum timing, duration of treatment, and preferred route of administration remained largely unanswered. We conducted a randomized controlled trial

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PATIENTS AND METHODS

Between January 1991 and June 1995, 142 patients with primary hepatocellular carcinoma underwent an elective hepatic resection at our institution. Our technique of hepatic resection has been described previously.[1] At the time of surgery, intraoperative ultrasonography was routinely performed to verify whether all macroscopic disease had been extirpated. For patients with no residual disease in the liver remnant, repeated imaging studies were conducted about 1 month after surgery. These included a percutaneous ultrasonographic examination and a hepatic angiogram. In the absence of any intrahepatic lesions on angiographic examination, iodized oil (Lipiodol, Lipiodol Ultrafluide, Laboratoire Guerbet, Aulnay-sous-Bois, France) was injected into the hepatic artery and this was followed up by a computed tomographic scan of the liver remnant 10 days later. The hepatectomy was considered curative only when these postoperative imaging studies demonstrated no residual tumors.

Seventy-six patients were excluded from this study for the following reasons: previous preoperative chemoembolization (8 patients), gross residual disease at the end of hepatic resection (19 patients), hospital mortality (9 patients), residual disease detected by imaging studies 1 month after undergoing an operation (30 patients), and refusal to participate (10 patients). Sixty-six patients who satisfied the criteria for a curative hepatectomy were enrolled in the study.

There were 53 men and 13 women with a mean age of 53.3 years (range, 28-78 years). The diameter of the tumor was more than 5 cm in 43 patients, 2 to 5 cm in 19 patients, and less than 2 cm in 4 patients. Forty-seven (71%) of 66 patients underwent major hepatectomy. Fifty-six patients (85%) were hepatitis B surface antigen–positive and 36 (55%) had cirrhosis of the liver on histologic examination. The mean interval between hepatectomy and randomization was 50 days (95% confidence interval, 40-59 days). All eligible patients were randomly assigned to receive either no treatment or postoperative adjuvant chemotherapy by drawing sealed consecutively numbered envelopes.

For patients assigned to receive postoperative adjuvant treatment, both systemic and transarterial chemotherapy were started immediately after randomization. Systemic chemotherapy consisted of a maximum of 8 doses of intravenous epirubicin hydrochloride (Pharmacia & Upjohn SPA, Milan, Italy), 40 mg/m^2 each, administered at 6-week intervals. In addition, 3 courses of transarterial chemotherapy were performed every 2 months via either 1 of the 2 routes. At the end of the operation for 24 patients (12 from each group) undergoing hepatic resection before September 1993, a cannula connected to a subcutaneous port (Implantofix, B. Braun Melsungen AG, Melsungen, Germany) was inserted into the gastroduodenal artery with its tip at the junction with the hepatic artery. This subcutaneous port provided atraumatic access to hepatic vasculature for angiography or transarterial chemotherapy when necessary. Alternatively, the hepatic artery supplying the liver remnant was selectively catheterized via the femoral artery under fluoroscopic guidance. Using the pumping method, an emulsion consisting of 10 mL of iodized oil and 10 mg of cisplatin (1 mg/mL) was prepared by mixing through a 3-way stopcock from one syringe to another. The emulsion was infused slowly into the hepatic artery until retrograde flow was evident. Intravenous or oral amoxicillin–clavulanic acid and cimetidine were administered immediately before the procedure and for 5 days afterward.

The primary end point was the occurrence of recurrent disease; the secondary end point was survival. The follow-up program was uniform for all patients and included a serum α-fetoprotein assay, chest radiograph, and percutaneous ultrasonographic examination of the liver remnant every 4 weeks for the first year and then at gradually increasing intervals. Suspected recurrent disease was confirmed with appropriate imaging studies and, if possible, histologic or cytologic examination. When recurrence was evident, adjuvant chemotherapy was stopped and the disease treated accordingly with treatment modalities such as reoperation, therapeutic transarterial chemoembolization, or systemic chemotherapy. No patient was lost to follow-up and all follow-up information was updated to May 31, 1996. The study protocol was approved by the Ethics Committee of the Faculty of Medicine of The University of Hong Kong and informed consent was obtained from each patient.

The necessary sample size required was estimated on the assumption that the incidence of recurrent tumor at the end of the third postoperative year for the control and treatment groups was 70% and 35%, respectively. Thirty-one patients were needed in each group to have a type I error of 5% and a type II error of 20% with a 2-tailed test.[14] Comparisons between groups were on an intention-to-treat basis. The statistical tests used included the Student t test, the Mann-Whitney U test, the χ^2 test with Yates correction, and the Fisher exact test where appropriate. The disease-free survival and survival rates were measured from the day of operation to the time when recurrent tumor was first localized by imaging studies and to the time of death, respectively. Survival was estimated according to the life-table method and was compared using the Wilcoxon test. Statistical significance was $P<.05$; all statistical analyses were conducted using a standard biomedical statistical program (SPSS/PC+, SPSS Inc, Chicago, Ill).

to define the benefits of postoperative adjuvant chemotherapy for patients who had a curative hepatectomy for hepatocellular carcinoma.

RESULTS

Thirty and 36 patients were randomized to the adjuvant chemotherapy and control groups, respectively. The 2 groups were comparable for sex, age, preoperative laboratory data, indocyanine green retention rate, tumor size, extent of resection, and operative blood loss (**Table 1**). The pathologic features of the resected specimens were also comparable (**Table 2**). The mean interval between hepatectomy and randomization was 47 days for the adjuvant chemotherapy group and 52 days for the control group ($P=.09$).

POSTOPERATIVE TRANSARTERIAL CHEMOTHERAPY

No complications were related to the insertion of the subcutaneous port in all 24 patients (12 from each group).

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Table 1. Clinical, Laboratory, and Operative Findings of 66 Patients Studied by Treatment Group*

Finding	Adjuvant Chemotherapy Group (n=30)	Control Group (n=36)
Sex (M/F)	26/4	27/9
Mean age, y (95% CI)†	54.6 (50.2-59)	53.4 (49.2-57.5)
Hepatitis B surface antigen–positive, No. of patients	25	31
Preoperative values		
Median α-fetoprotein titer, ng/mL (range)	246.5 (1-735 000)	181.0 (1-388 800)
Mean serum total bilirubin, μmol/L [mg/dL](95% CI)	8.63 [0.5](7.52-9.75)	13.23 (8-18.45)
Mean serum albumin, g/L (95% CI)	43.7 (42-45.5)	43.8 (42.1-45.6)
Mean prothrombin time, s>control (95% CI)	0.49 (0.29-0.68)	0.63 (0.3-0.96)
Mean indocyanine green retention rate at 15 min, % (95% CI)	11.1 (9.9-12.3)	11 (8.8-13.3)
Tumor size		
Mean, cm (95% CI)	8.5 (6.8-10.1)	10.4 (5.2-15.6)
>5 cm, No. of patients	20	23
Major hepatectomy, No. of patients	23	24
Operative blood loss, L (95% CI)	2.1 (1.6-2.6)	2.3 (1.8-2.8)

**All variables are statistically comparable between the 2 groups.*
†CI indicates confidence interval.

The port failed to provide vascular access owing to occlusion or malposition in the early postoperative period in 9 patients (38%), 4 of whom were in the adjuvant chemotherapy group. Three of these 4 patients had successful transarterial chemotherapy performed via the femoral artery, but the remaining 1 had a thrombosed hepatic artery precluding any transarterial injection. Thus, 29 of 30 patients received transarterial chemotherapy via the subcutaneous port (8 patients) or the transfemoral route (21 patients). Fifteen patients received all 3 courses of treatment, while adjuvant transarterial chemotherapy was discontinued in the remaining 14 patients because of recurrent disease (12 patients) and refusal to continue (2 patients).

Three patients had local complications after transarterial chemotherapy via a subcutaneous port. Two patients had cellulitis from extravasation around the port and 1 had severe epigastric pain with necrosis of the lesser curve of the stomach shown on endoscopy. All 3 patients were treated conservatively and, except for 1 who had thrombosis of the hepatic artery, were able to continue treatment via the transfemoral route. There were no other serious adverse effects, such as liver failure, from the transarterial chemotherapy and no patient had any local complications as a result of the femoral artery catheterization.

POSTOPERATIVE SYSTEMIC CHEMOTHERAPY

One hundred and thirty-eight courses of intravenous epirubicin were given to 30 patients in the adjuvant chemotherapy group. Eleven patients received all 8 planned courses but in 19 patients treatment was stopped because of recurrent disease (15 patients), adverse effects (2 patients), and refusal to continue (2 patients). Adverse reactions were recognized in 3 patients during the administration of systemic chemotherapy. One patient with a previous history of thyrotoxicosis had atrial fibrillation and was treated with digoxin. Another patient had leukopenia (lowest white blood cell count, 1.98 × 10^9/L) and recovered uneventfully. In both cases, systemic chemotherapy was stopped. The remaining patient had alopecia, which did not affect the schedule of the adjuvant treatment. Thus the overall complication rate for adjuvant transarterial and systemic chemotherapy was 20% (6 of the 30 patients) and there was no treatment-related mortality.

Table 2. Pathologic Features of 66 Patients Studied by Treatment Group*

Feature	Adjuvant Chemotherapy Group (n=30)	Control Group (n=36)
Cirrhosis	17	19
Multinodular lesion	11	15
Mean macroscopic resection margin, cm (95% CI)†	1.39 (0.96-1.82)	1.45 (1-1.87)
Positive histologic margin	1	5
Venous permeation	14	16
Microsatellite	10	15
Encapsulation	14	24
Capsular invasion	7	14
Stage		
I	1	3
II	9	12
III	20	21

**All variables are statistically comparable between the 2 groups. Stage of tumor is classified according to the description of the Liver Cancer Study Group of Japan.[15]*
†CI indicates confidence interval.

RECURRENT DISEASE AND DISEASE-FREE SURVIVAL

At a median follow-up time of 28.3 months (range, 4.9-77.1 months), 23 of the 30 patients in the adjuvant chemotherapy group and 17 of the 36 patients in the control group had proved recurrent disease ($P=.01$). Recurrence in the liver remnant alone was found in 24 patients, in extrahepatic organs alone in 8 patients, and in both sites in 8 patients (**Table 3**). There was no difference in the incidence of intrahepatic recurrence be-

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Table 3. Sites of Recurrence by Treatment Group

Sites of Recurrence	Adjuvant Chemotherapy Group (n=30)	Control Group (n=36)
Intrahepatic	16	16
Extrahepatic	11	5†
Lung	9	5
Bone	2	0
Total (either site)*	23	17‡

**Four patients from each group had both intrahepatic and extrahepatic recurrences.*
†P=.03 compared with adjuvant chemotherapy group.
‡P=.01 compared with adjuvant chemotherapy group.

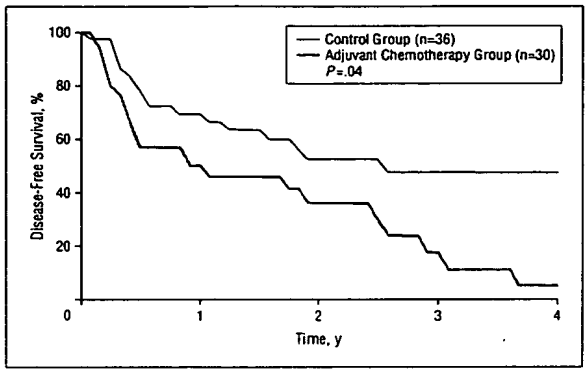

Figure 1. *Disease-free survival curves after curative resection of hepatocellular carcinoma. Patients who had adjuvant chemotherapy had a lower disease-free survival than those in the control group (P=.04).*

tween the 2 groups but patients who received adjuvant chemotherapy had more extrahepatic recurrences (11 patients) than those who were assigned to the control group (5 patients) ($P=.03$). The disease-free survival of the adjuvant chemotherapy group was worse than that of the control group ($P=.04$). The respective 1-, 2-, and 3-year disease-free survival rates were 50%, 36%, and 18% for patients in the adjuvant chemotherapy group and 69%, 53%, and 48% for patients in the control group (**Figure 1**).

Among the 40 patients with recurrent disease, 12 patients received therapeutic transarterial chemoembolization for intrahepatic recurrence, 22 patients received systemic chemotherapy (with additional external radiotherapy for spinal metastases in 2 patients), and 5 patients were treated symptomatically because of poor performance status. One patient had resection of a solitary pulmonary metastasis followed by intravenous epirubicin treatment and remained disease-free at 3 years after the second operation.

SURVIVAL

At the time of analysis, 10 of the 30 patients in the adjuvant chemotherapy group and 10 of the 36 in the control group had died. The cause of death was progressive recurrent hepatocellular carcinoma in all patients except 1, who died of an unknown cause in the absence of

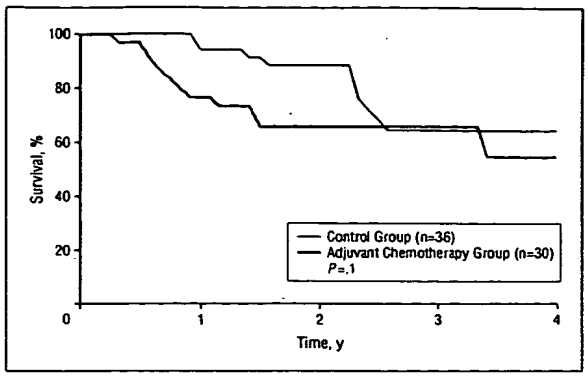

Figure 2. *Survival curves after curative resection of hepatocellular carcinoma. The difference in survival between the 2 groups was not statistically significant (P=.10).*

any evidence of recurrence. The survival of patients assigned to the treatment group was worse than that of the control group (**Figure 2**), particularly in the first 2 years after the operation, although the difference was not statistically significant ($P=.10$).

COMMENT

When considering postoperative adjuvant chemotherapy that aims primarily at preventing tumor recurrence, the distinction between recurrent disease after a curative operation and residual tumor after a palliative resection is crucial. The curability of hepatectomy for hepatocellular carcinoma is difficult to define. The definition based on tumor staging and resection margin recommended by the Liver Cancer Study Group of Japan[15] is so restrictive that few resections included in the present study could be considered curative. In addition, it does not consider tiny intrahepatic metastases that are not detected by preoperative imaging studies or intraoperative ultrasonography. We have adopted the definition, as described by Nagasue et al,[11] that in addition to preoperative and intraoperative findings, a hepatectomy is considered curative only when imaging studies conducted about 1 month after surgery do not reveal any residual disease. Thus, only patients with no demonstrable tumor at the time of randomization are considered suitable for adjuvant chemotherapy, whereas those with residual disease discovered by such screening immediately after operation should be treated therapeutically. Although there are limitations in current liver imaging techniques,[16] a thorough intraoperative ultrasonographic examination followed by repeated investigations using ultrasonography, angiography, and post-Lipiodol computed tomography is regarded as the most sensitive means to confirm the absence of any demonstrable intrahepatic disease before initiation of adjuvant chemotherapy. Even so, more than 50% of the patients in the control group had recurrence at 3 years and the need for adjuvant treatment was justified.

The optimum route of administration, exact regimen, and timing of adjuvant chemotherapy is uncertain. Although the hepatic remnant is the predominant site of recurrence, involvement of extrahepatic organs such

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as the lung and bone are frequent.[4,8] Our previous study of 277 patients who underwent hepatic resections for hepatocellular carcinoma showed that 25.8% had extrahepatic recurrences.[8] The lower rate of extrahepatic recurrence of 13.9% (5 of the 36 patients) in the control group of the present study can be explained by the definition of a curative hepatectomy, which excludes any patient with residual or recurrent disease within the first month of surgery. Transarterial chemotherapy is an effective locoregional therapy for unresectable[17-19] or recurrent hepatocellular carcinoma[20] and recent nonrandomized studies showed that it might reduce intrahepatic recurrences after hepatic resection.[10,12,13] However, this regional therapy is of no value for extrahepatic tumor dissemination. For adjuvant treatment to be effective, it is conceivable that postoperative chemotherapy should be provided transarterially and systemically.

Based on our experience[17] and that of others[19] in unresectable hepatocellular carcinoma, the response rate of transarterial chemotherapy using an emulsion of iodized oil and cisplatin is between 38% and 55% and may be better than that of treatment using iodized oil and doxorubicin.[19] In the absence of any demonstrable tumor, a maximum dose of 20 mL of the emulsion was considered adequate. Takenaka and associates[13] recommended postoperative lipiodolization only once or twice, but in view of the high risk for intrahepatic recurrence, the present regimen was intensified to 3 courses of treatment within 6 months. As for systemic chemotherapy, doxorubicin is one of the most active drugs against hepatocellular carcinoma, with a response rate of 10% to 24% in patients with advanced disease.[21-24] Hence, its derivative epirubicin was used because of its reduced cardiac toxic effects. Previous experience with 3 doses of intravenous epirubicin hydrochloride every 3 weeks at full strength (75 mg/m^2) after hepatectomy for large tumors showed a high incidence of drug-induced toxic effects, particularly hepatic decompensation.[25] We therefore administered epirubicin hydrochloride at half-doses (40 mg/m^2) at longer intervals, up to a maximum of 320 mg/m^2 over 1 year in the present study.

With regard to the timing of adjuvant chemotherapy, Takenaka and associates[13] started adjuvant transarterial chemotherapy for their patients more than 1 year after surgery. In contrast, other investigators would start at 2 to 6 weeks after surgery and repeat every 3 months for 1 year or longer.[10,12] The rationale of administering adjuvant chemotherapy after curative resection is to prevent recurrence by suppressing microscopic neoplastic foci. Furthermore, it was reported that recurrence after hepatic resection for hepatocellular carcinoma was most common within the first postoperative year,[1,7] and this is true even in the present series of selected patients with curative resection. Adjuvant chemotherapy for hepatocellular carcinoma therefore should be started soon after resection. Theoretically, the administration of either regional or systemic chemotherapy soon after hepatic resection may affect the performance status of the patient and depress the regenerative activity of the liver remnant, particularly if there is underlying liver cirrhosis. We withhold chemotherapy for at least the first 4 weeks after the operation and with the present regimen, combined adjuvant transarterial and systemic chemotherapy seems to be safe with no serious complications when administered to patients starting 6 to 8 weeks after hepatectomy.

Both surgical cannulation of the hepatic artery followed by placement of a subcutaneous port and transfemoral arterial puncture had been used successfully for delivering chemotherapy intra-arterially.[10-13] The subcutaneous port was used in the early part of our study because of its theoretical advantage of providing an atraumatic means for repeated access to the hepatic vasculature. Nevertheless, the additional operation time, frequent early occlusion, and morbidity associated with these devices had made the transfemoral route our preferred means for intra-arterial drug administration. The latter route had been employed successfully in all 21 patients without a functioning subcutaneous port with minimal morbidity.

Recent retrospective studies have shown encouraging results with adjuvant transarterial chemotherapy following hepatic resection for hepatocellular carcinoma. Using a combination of fluorouracil, doxorubicin, and mitomycin in Lipiodol delivered transarterially, Nonami and associates[10] found a better survival rate in 19 patients who were treated after the operation than 113 who were not. According to Nagasue et al,[11] a significant survival benefit was obtained by giving their patients intravenous epirubicin and peroral fluorouracil after hepatectomy. In a prospective nonrandomized study, Takenaka et al[13] found a significantly higher disease-free survival in patients who received lipiodolization after hepatectomy than others receiving no treatment, although the timing of their initiation of treatment varied widely from less than 6 months to almost 2 years after the operation. Without a proper control group, these studies had a serious drawback of patient-selection bias. In addition, without proper documentation of a curative resection and the absence of residual disease before initiation of adjuvant treatment, it is difficult to know whether the beneficial effect on survival is merely related to early therapeutic intervention for residual or recurrent disease.

A prospective randomized controlled trial[26] showed improved disease-free survival and overall survival with the use of oral 1-hexylcarbomoyl-5-fluorouracil following curative resection for hepatocellular carcinoma. The study, however, involved 26 institutions with a mean of only 2.3 inclusions per institution. The favorable results of this adjuvant chemotherapy trial may be questionable because treatment was suspended owing to adverse effects in 12 (44%) of 27 patients. In contrast, the present randomized controlled study showed that combined transarterial and systemic adjuvant chemotherapy using the present regimen has compromised the disease-free survival and probably the overall survival of a selected group of patients with curative resection of hepatocellular carcinoma. The possibility that angiographic studies performed during transarterial chemotherapy resulted in earlier detection of recurrences and hence a shorter disease-free survival is unlikely. Instead of improving the survival by this early detection of recurrences, the survival of the treatment group was lower, largely because of a higher incidence of extrahepatic metastases and cancer death.

The exact reason for the negative result observed is open to speculation. First, transarterial chemoemboli-

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zation was associated with a higher incidence and earlier development of extrahepatic metastases in patients with unresectable hepatocellular carcinoma.[18,27] The defective blood vessels or the ingrowth of new blood vessels in zones of tumor necrosis may facilitate systemic tumor dissemination. Second, definite evidence shows that malignant primary tumors contain subpopulations of cells that are heterogeneous for metastatic potential and susceptibility to cytotoxic drugs.[28] By destroying the subpopulation of drug-sensitive cells, chemotherapy could stimulate the formation of new clonal variants from the surviving subpopulations[29] and permit cells with a higher metastatic capability to proliferate. Finally, immune surveillance in control of tumor dissemination may be incriminated. The antimitotic effect of the present regimen of adjuvant chemotherapy might have depressed the host immunity against tumor metastasis.[30]

The failure of adjuvant chemotherapy in the present study may call for consideration to intensify the therapeutic regimen. Nevertheless, limitations are inherent in any form of chemotherapy for hepatocellular carcinoma not only because many tumors are slow growing[30] and hence cytotoxic drug–resistant, but also because the associated liver cirrhosis limits the maximum tolerated intensity of chemotherapy. Further prospective studies using other regimens are required before the value of postoperative adjuvant chemotherapy can be defined more clearly.

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Surgical Anatomy

The sympathetic trunk is composed of ascending and descending fibers, some of which are preganglionic efferent, postganglionic efferent, and afferent fibers.

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Cancer. 1988 Aug 1; 62(3): 479-83.

Doxorubicin versus no antitumor therapy in inoperable hepatocellular carcinoma. A prospective randomized trial.

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To assess the efficacy and safety of Adriamycin (Adria Laboratories, Columbus, OH) in inoperable hepatocellular carcinoma (HCC), 60 patients were randomized to receive Adriamycin 60 to 75 mg/m2 at 3-week intervals and 46 patients to receive no antitumor therapy. The median survival rate of the Adriamycin group was 10.6 weeks; that of the group receiving no antitumor therapy was 7.5 weeks (P = 0.036). Adriamycin induced tumor regression of 25% to 50% in 5% of patients and of over 50% in only 3.3% of patients. It caused fatal complications (septicemia and cardiotoxicity) in 25% of patients. The severity of neutropenia leading to septicemia for a particular dose was unpredictable. Four of eight patients who developed cardiotoxicity received less than 500 mg/m2 of Adriamycin. We conclude that Adriamycin is not an ideal drug for the treatment of inoperable HCC.

Publication Types:

- Clinical Trial
- Randomized Controlled Trial

PMID: 2839280 [PubMed - indexed for MEDLINE]

Reg Cancer Treat 3(4): 197-201, 1990

Treatment of primary hepatocellular carcinoma by hepatic arterial infusion of 4'-epirubicin (Eng).

Shepherd FA; Rotstein L; Blackstein ME; Burkes R; Erlichman C; Iscoe N; Kutas G; et al:

A group of 23 patients (20 male, 3 female) with hepatocellular carcinoma were treated by hepatic arterial infusion of 4'-epirubicin every 4 weeks. At each treatment, a catheter was inserted percutaneously into the main hepatic artery via the femoral artery under image intensification. Treatment consisted of a 24-h continuous HAI of epirubicin, 30 mg/msup 2/day for 3 days, without heparin. Eleven patients had only one infusion, 4 patients two infusions, 2 patients three infusions, 2 patients four infusions, and 1 patient six and eight infusions each. A partial response was seen in 3 patients, median duration 16 weeks (range 12-46 weeks). Seven patients remained stable, median duration 13 weeks (range 4-38 weeks). The median survival of the overall group was 18 weeks. Survival of responding, stable, and non-responding patients were 38 weeks, 19 weeks, and 10 weeks, respectively. Complications of catheter placement included asymptomatic dissection of the hepatic artery (3 patients), and asymptomatic thrombosis of the hepatic artery (3 patients). Eight patients experienced moderate nausea and vomiting, and 11 patients had moderate to severe alopecia. The granulocyte nadir was above 1000 mul in 83% of evaluable courses, 500-1000 mul in 6%, and less than 500 mul in 11% of courses. Two patients developed neutropenia-associated fever. A platelet nadir below 100,000/mul was seen after only 8% of courses, and only 1 patient had platelets below 50,000/mul. In conclusion, epirubicin has modest activity in hepatocellular carcinoma and is well tolerated when given by hepatic arterial infusion.

(Meeting abstract) (Eng). Ann Oncol 5(Suppl 8)1994

Arterial chemoembolization with epirubicin in unresectable hepatocellular carcinoma in cirrhosis

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No reliable therapies have yet been established for unresectable hepatocellular carcinoma (HCC). Systemic chemotherapy with anthracyclines gives less than 20% objective remissions. Encouraging data in terms of response rate and survival have recently been reported with intra-arterial chemotherapy alone or combined with various veno-occlusive materials, specifically ethiotized oil and gelatin sponge; To evaluate the activity and tolerance of a new chemoembolization protocol, patients with unresectable HCC in cirrhosis were treated with epirubicin (50 mg) and ethiotized oil (10-15 ml), administered through hepatic arterial catheters placed percutaneously during angiography, followed by gelatin sponge. Therapy was repeated for a maximum of 3 cycles. Twenty-two eligible patients have entered the study and are evaluable for response and toxicity. Patients were not pretreated with chemotherapy, and only 1 patient had been submitted to surgery. Patient characteristics were: median ag 70 yr (range 59-77); ECOG performance status 0-1 in 15 and 2 in 7 cases; Child's A disease in 11 and B in 11; Okuda Stage I in 12 and Stage II in 1 cases; TNM Stage II in 9, Stage III in 3 and Stage IVA in 10 cases. Histologically documented cirrhosis was present in all cases (10 alcohol correlated, 4 Hb-sAg correlated, and 8 HCV related).

A total of 53 courses of therapy have been delivered. Three partial remissions (13%), 2 stabilizations of disease and 17 progressions have been observed. Median time to progression was 4 mo with a median survival of 7.6 mo (range, 1-26+ mo). Significant differences in survival (p less than 0.0001) have been observed between patients at Stage II-III (20 mo) and those at stage IVA (3 mo). The treatment was well tolerated with only 2 cases of WHO Grade I pain and 2 cases of Grade I fever. In conclusion, our results indicate that the schedule has only limited activity in HCC and does not seem to offer any sure advantage over other treatments modalities in HCC.